



Impact of Resolvin E1 on Experimental Periodontitis and Periodontal Biofilm

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Impact of Resolvin E1 on Experimental Periodontitis and Periodontal Biofilm

A Thesis Presented by

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to

The Faculty of the Harvard School of Dental Medicine

in partial fulfillment of the requirements

for the degree of

Doctor of Medical Sciences in Oral Biology

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DEDICATION

To my parents, my wife, and family for their love and support

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ABSTRACT

Objective: The goal of this project was to determine the impact of local inflammation on changes in the subgingival biofilm composition in ligature-induced periodontitis in rats using the specialized pro-resolving mediator (SPM), resolvin E1 (RvE1).

Materials and Methods: The impact of RvE1 on the microbiota of ligature-induced periodontitis was assessed in two separate experiments; treatment of established periodontitis and prevention of ligature-induced periodontitis. In the treatment study, eighteen rats were separated into four groups comprising no ligature, ligature alone (no treatment), ligature with topical RvE1 treatment (ligature+RvE1) and, ligature with topical vehicle treatment (ligature + Vehicle). 3-0 silk ligatures were tied around maxillary second molars bilaterally for three weeks to induce disease. After three weeks, the treatment phase began with the application of RvE1 or vehicle (ethanol) every other day for an additional three weeks. Subgingival plaque samples were collected every four days throughout the experiment. The composition of the subgingival microbiota was initially screened by checkerboard DNA-DNA hybridization using probes on 40 subgingival species. Definitive, unbiased characterization of the subgingival microbiota was accomplished with next-generation sequencing using the Illumina MiSeq® platform. Six rats were sacrificed on Days 1, 21 and 42 and maxillae were dissected to collect samples for gingival RNA extraction, bone morphometric measurements, and histomorphometric analysis. Local tissue gene expression (*Cxcl1*, *Ptgs2*, *Nos2*) was detected using qRT-PCR. Tissue specimens were prepared for histology and stained with

H&E and tartrate resistant acid phosphatase (TRAP). In the prevention study, sixteen rats were separated into four groups (no ligature, ligature + RvE1 (0.1 µg/µl), ligature + RvE1 (0.5 µg/µl), ligature + Vehicle). 5-0 silk ligatures were placed around maxillary second molars bilaterally to induce disease. At the time of ligature placement, animals received assigned treatment thrice weekly (M, W, F) for four weeks. Subgingival plaque samples were collected every four days (M and F). Four rats were sacrificed at baseline (Day 1) and the vehicle and two treatment groups (four each) were sacrificed at day 28 and samples processed as described above. The two-group comparisons were assessed by Student's t-test. The multiple-group comparison was assessed by one-way ANOVA and post hoc tests.

Results: In the first study (treatment), topical application of RvE1 significantly reversed the bone loss associated with periodontitis compared to the vehicle. RvE1 application significantly reduced the expression of *Cxcl1* and osteoclast density compared to the vehicle application. In the prevention study, RvE1 treatment significantly prevented the bone loss during the disease progression. RvE1 application significantly reduced the expression of *Ptgs2*, *Nos2* compared to the vehicle application. Osteoclast density and inflammatory cell infiltration in the RvE1 groups were significantly lower than these in the Vehicle group.

The cell counts of bacterial species gradually increased and the subgingival microbiota shifted during the disease progression. In the treatment study, RvE1 treatment significantly reduced cell counts compared to the vehicle application at the end of treatment phase. The shift of subgingival microbiota was limited by the RvE1 treatment.

In the prevention study, the taxonomic composition and diversity of subgingival microbiota was controlled by the RvE1 application. The change of subgingival microbiota appeared to be associated with the state of inflammation in the periodontal environment.

Conclusion: Resolvin E1 treatment of existing ligature-induced periodontitis significantly regenerates lost alveolar bone and prevents alveolar bone loss. Resolvin E1 treatment limits microbial shifts and reduces total bacterial load by inhibiting inflammation of local environment in experimental periodontitis.

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CHAPTER ONE: BACKGROUND

Periodontitis

Chronic periodontitis is a multifactorial inflammatory disease with high prevalence in different populations. In the US, around 64 million over 30 year-old adults have chronic periodontitis (47.2%). The prevalence of chronic periodontitis is higher than the prevalence of diabetes (Eke et al., 2012). Chronic periodontitis has been associated with several systemic inflammatory diseases, including rheumatoid arthritis, Type 2 diabetes and cardiovascular diseases (Demmer and Papapanou, 2010, Kebschull et al., 2010, Lalla and Papapanou, 2011). These inflammatory diseases share similar pathological mechanisms, such as an accumulation of immune cells and production of excessive pro-inflammatory cytokines that lead to unresolved inflammation. Infection with (putative) bacterial pathogens in a susceptible host has been considered the primary etiological factor of periodontal disease. Although some periodontal pathogens produce enzymes damaging periodontal tissues directly, the immune responses, including production of pro-inflammatory cytokines, reactive oxygen species (ROS) and collagen-dissolving enzymes, induced by these bacterial species are the major causes of periodontitis (Hausmann et al., 1972, Stashenko et al., 1987, Cekici et al., 2014, Jiao et al., 2014). Several cultivable bacterial species, such as *Porphyromonas gingivalis* (*P.gingivalis*), *Tannerella forsythia* (*T. forsythia*) and *Treponema denticola* (*T. denticola*), have been shown to induce alveolar bone loss around teeth in animal models (Lalla et al., 1998, Schreiner et al., 2003, Hasturk et al., 2006), and are correlated with severity of periodontitis clinically (Socransky et al., 2004, Paster et al., 2006). The composition of

the microbiota in health and periodontitis is quite different (Teles et al., 2006). With the advent of molecular methodologies to identify yet uncultured bacteria, it was realized that the number and diversity of microorganisms in the subgingival microbiota were greater than previously appreciated. Likewise, the complexity of the host immune response to the biofilm was also under appreciated. It is now known that different species induce distinct immune responses in animal models of periodontitis (Gemmell et al., 2004, Kopitar et al., 2006). There is no specific bacterial profile or specific bacteria generally associated with periodontitis in every patient (Pihlstrom et al., 2005). Although it is debatable whether specific pathogens initiate periodontal diseases, the effect of reducing bacteria on controlling periodontal disease is undeniable (Socransky and Haffajee, 2005). Adjunctive use of antibiotics with periodontal scaling and root planing improves clinical outcomes of periodontal treatment (Goodson et al., 2012, Feres et al., 2012). The change in the subgingival microbiota accompanying the improvement of the clinical outcomes with antibiotic use has also been demonstrated (Soares et al., 2014).

The periodontium comprises gingiva, cementum, periodontal ligament, and alveolar bone. Histologically, the pathogenesis of periodontitis is characterized by stages, including the initial lesion, the early lesion, the established lesion, and the advanced lesion (Page and Schroeder, 1976). The initial lesion starts within 24 hours after dental plaque is deposited on the tooth's surface. Dilation of the arterioles, capillaries and venules of the dentogingival plexus is evident underneath the junctional epithelium. Exudate forms in the gingival tissue, because the permeability of the microvascular bed increases. As intercellular gaps in the epithelium become larger with increased

inflammation, the exudate, which is called gingival crevicular fluid, seeps into the gingival sulcus. Unattached bacteria are flushed away and plasma proteins, such as antibodies, complement, and proteinase inhibitors in gingival crevicular fluid can have an impact of the composition of the biofilm. Neutrophils consistently appear adjacent to the epithelium of gingival sulcus, which is essentially an open environment routinely challenged by bacteria and food debris. Eventually neutrophils migrate out of the junctional epithelium into the sulcus following a chemoattractant gradient of bacterial peptides. These activated neutrophils express selectins and intracellular adhesion molecules on their surfaces that bind to upregulated adhesion molecules on junctional epithelial cells (Moughal et al., 1992).

In the next stage, the early lesion, the vessels underneath the junctional epithelium remain dilated, and the number of vessels increases. The increased size and quantity of microvasculature units are reflected in the clinical redness of the gingival margin (Lindhe and Rylander, 1975). Gingiva tends to bleed easily when a periodontal probe is placed into the sulcus. Gingival redness and bleeding on probing are the first clinical signs of gingivitis. Lymphocytes and neutrophils are the predominant cells infiltrating in the connective tissue and very few plasma cells are observed within the lesion at this stage (Seymour et al., 1983, Brex et al., 1987). Fibroblasts in the lesion degenerate and collagen fibers are damaged with cell apoptosis and production of matrix metalloproteinases by inflammatory leukocytes and resident stromal cells. The loss of collagen fibers permit additional leukocyte infiltration (Page and Schroeder, 1976, Takahashi et al., 1995). The basal cells of the junctional and sulcular epithelium start to

proliferate rapidly. The proliferation of these cells represents an attempt to enhance the mechanical barrier to block bacteria and bacterial products. Epithelial rete pegs become elongated and invade the coronal portion of the lesion (Schroeder, 1970). The start of early lesion was seen as early as one week after plaque accumulation and could persist for weeks. The required time for the early lesion to convert to the next stage, the established lesion, was hypothesized to depend on host susceptibility and virulence of bacteria.

The established lesion is an enhanced inflammatory lesion compared to the early lesion. Increased fluid exudation and leukocyte migration into the tissues and the gingival sulcus are observed. The tissues are more swollen clinically compared to the early lesion. The loss of collagen and the proliferation of epithelium continue and even more leukocytes infiltrate the connective tissue. The diseased epithelium is more permeable and bacterial products can pass into the connective tissue more easily. The established lesion is dominated by plasma cells (Page and Schroeder, 1976). In the gingival biopsies of adults younger than 30, (Brecx et al., 1988, Fransson et al., 1996), lymphocytes occupy a larger area in the lesion than plasma cells. However, plasma cells are dominant in the established lesion of gingival biopsies from subjects who are over 65 years old (Fransson et al., 1996). The established lesion may persist for months or years and never progress to a more advanced lesion.

The final stage of progression is known as the advanced lesion. The first three stages are considered gingivitis, but the advanced lesion is periodontitis. In the advanced lesion, the elongation of junctional epithelium, the destruction of collagen, and the

infiltration of leukocytes continue. The changes in the tissues, including a deepening of the pocket, excessive inflammation and anaerobiosis, establish a habitat for the growth of periodontitis associated Gram-negative anaerobic bacteria considered pathogens (Socransky and Haffajee, 2005). The most distinct characteristic of the advanced lesion is the loss of connective tissue attachment to the tooth and the destruction of alveolar bone. Periodontitis is clinically distinct from gingivitis. Generally, it is accepted that plasma cells are the dominant cell type in the advanced lesion (Grant and Mulvihill, 1972, Berglundh and Donati, 2005).

In summary, the dynamics of periodontitis pathogenesis is well established from a myriad of observations. However, the timing of transition from one lesion to another really depends on the individual and is affected by many factors, such as systemic health, oral hygiene habits, smoking, and other factors, both modifiable and non-modifiable. Severe gingivitis characterized by the established lesion increases risk for periodontitis, but it may never progress to an advanced lesion.

Treatment of Periodontitis

Mechanical debridement has been the standard treatment of periodontitis for decades (Heitz-Mayfield and Lang, 2013, Jan Lindhe, 2008). Mechanical debridement usually starts with scaling and root planing, which is a procedure designed to clean calculus from crown and root surfaces using special instruments without surgically elevating gingival tissues. A surgical approach is sometimes required when scaling and root planing alone does not generate positive outcomes usually due to inadequate access. Elimination of bacterial plaque and calculus is the goal of mechanical debridement. It is

believed that removal of subgingival plaque is sufficient to reduce the immune response stimulated by bacteria and stop tissue destruction. However, subgingival debridement has efficiency limitations and cleansing of diseased areas is often incomplete (Slots, 2002). Regardless, of completeness of debridement, bacteria grow back gradually after the mechanical therapy in the absence of further treatment or maintenance (Haffajee et al., 2006). Adjunctive use of antibiotics improves clinical outcomes, but antibiotic resistance and other side effect are always a concern. In refractory periodontitis patients, severe periodontal tissue destruction is seen often despite relatively low bacterial challenge, appropriate periodontal maintenance and treatment (Teles et al., 2010).

In recent years, it has become apparent the pathogenesis of periodontal diseases is mediated by the host and that susceptibility to disease is a function of the inflammatory response (Van Dyke, 2011, Van Dyke, 2014). Subjects with severe periodontitis exhibit measurable excess inflammation that includes excess cytokine production (Graves, 2008) and oxidative stress (Kantarci et al., 2003, Chapple and Matthews, 2007). It is also now realized that there is little if any tissue destruction that is the direct result of bacterial enzymes or toxins. The presence of, or number of, specific bacteria do not predict disease, but inflammation does (Tanner et al., 2007). Efforts to control the host inflammatory response have shown that disease can be arrested with COX inhibitors without modifying bacteria (Williams et al., 1989, Howell, 1993, Jeffcoat et al., 1995), but these treatments are not safe long term. Hence now, considerable effort is now being devoted to the development of rational and safe host modulation therapies to prevent and treat periodontitis.

Resolution of Inflammation and Specialized Pro-Resolving Mediators (SPMs)

In addition to the acute pro-inflammatory phase, “resolution” is an active phase of the inflammatory process first described and extensively characterized by Charles N. Serhan (Serhan, 2014). Failure to resolve inflammation leads to chronic inflammation, which causes tissue damage and induces disease. It is now realized that inflammatory disease may be a failure of resolution rather than overproduction of pro-inflammatory mediators (Serhan et al., 2008). The resolution response reduces tissue damage, removes cell debris and helps tissues return to homeostasis (Serhan and Savill, 2005). Specialized pro-resolving mediators (SPMs) play an important role in the resolution phase of acute inflammation.

These SPMs, including lipoxins (LXA4, LXB4), resolvins (RvE, RvD), protectins (PD), and maresins (MaR), are derived from ω -6 [arachidonic acid], and ω -3 [eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA)] polyunsaturated fatty acids, respectively (Serhan, 2010) (Fig. 1.1). The ω -3 fatty acids are found in marine oils and have been recognized having anti-inflammation properties (Albert et al., 2002, Calder and Yaqoob, 2009). However, the molecular mechanism behind the anti-inflammation properties was not clear until the metabolic products of ω -3 fatty acids, resolvins, protectins and maresins, were described. These molecules were first found in the exudates of the acute inflammation phase in a murine model (Serhan et al., 2000). In the process of acute inflammation, lipids in the cells and tissues are either transformed to pro-inflammatory lipid mediators, such as prostaglandins (PGD2, PGE2) and leukotrienes (LTC4, LTB4), or SPMs (Serhan et al., 2009, Serhan, 2014). The pathway of

lipid mediators changing from pro-inflammatory molecules to pro-resolution molecules is called “class switch” (Levy et al., 2001, Serhan et al., 2007). The process of class switch is triggered by lipoxygenases (LO) and other enzymes, such as aspirin acetylated cyclooxygenase-2 (COX2), which becomes a 15R-lipoxygenase, or cytochrome P450, during cell-cell interactions. The importance of these enzymes to initiate the production of SPMs and activate resolution of inflammation has been demonstrated in many studies. For example, the overexpression of 15-lipoxygenase in the transgenic rabbit model increased available arachidonic acid derived substrate (15-HETE) for 5-LO leading to increased circulating LXA4 and increased resolution of inflammation (Shen et al., 1996, Serhan et al., 2003). Moreover, aspirin acetylated COX-2 produces similar products from EPA and DHA, and these intermediates can be transformed by human neutrophils *in vitro*. This class of compound is collectively known as aspirin-triggered lipoxins or aspirin-triggered resolvins (Serhan et al., 2000, Serhan et al., 2002).

In the process of class switch, different cell-cell interactions, such as leukocytes and leukocytes, leukocytes and platelets, leukocytes and epithelial cells, produce SPMs through different pathways of transcellular biosynthesis (Romano and Serhan, 1992, Gronert et al., 1998, Wallace and Fiorucci, 2003, Tian et al., 2009). Lipid mediator class switching is induced significantly during inflammation and this process has been observed in humans doing resistance exercise (Markworth et al., 2013).

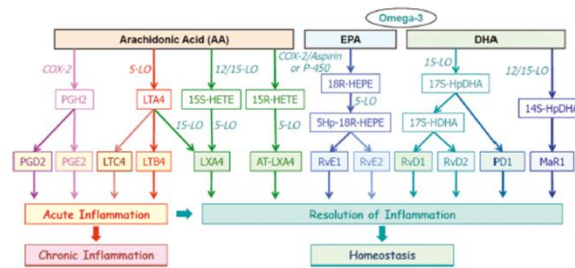


Figure 1.1 Biosynthetic cascades and actions of specialized pro-resolving mediators (from Serhan & Petasis 2011)

In the resolution phase, the SPMs can reduce neutrophil infiltration, promote neutrophil apoptosis (El Kebir et al., 2012), and recruit non-phlogistic macrophages (Oh et al., 2011, Oh et al., 2012, Stables et al., 2011) which perform efferocytosis (Serhan et al., 2008). These activities can clean up the “battlefields” of inflammation to restore the original biologic-architecture following acute inflammation. In addition to the impact of SPMs on innate immunity, adaptive immunity is also regulated by SPMs, including inhibiting Th17 cell response, activating natural killer (NK) cell homing, activating NK cell mediated clearance of antigen specific T cells and eosinophils in allergic inflammation (Levy, 2012), reducing T cell migration and production of interferon- γ , tumor necrosis factor- α (TNF- α) (Ariel et al., 2005), stimulating chemokine scavenging from apoptotic T-cells in peritonitis model (Ariel et al., 2006), and stimulating human B cell differentiation (Ramon et al., 2012).

SPMs bind to G-protein-coupled receptors (GPCRs) on cells to activate the functions. Several SPM receptors, including leukotriene B4 receptor 1 (BLT1), Chemokine like receptor 1 (ChemR23), formyl peptide receptor 2 (ALX/FPR2), G protein-coupled receptor 32 (GPR32), have been identified on a variety of cell types. For example, resolvin E1 (RvE1) specifically binds to receptors BLT1 on the neutrophils and

ChemR23 on macrophages to evoke pro-resolving responses (Arita et al., 2005a). The binding of RvE1-BLT1 also blocks the LTB4-BLT1 binding which activates the survival signaling in neutrophils. RvE1 activation of ChemR23 can enhance macrophage phagocytosis by phosphoprotein-mediated signaling (Ohira et al., 2010).

In addition to regulating the immune response, SPMs also actively play the role in the wound healing and tissue regeneration. Resolvin D1 (RvD1) and D2 (RvD2) are able to stimulate wound healing in diabetic mice by inducing resolution of inflammation (Tang et al., 2013). RvE1 and Maresin 1 (MaR1) both can reduce time required for regeneration of head segment in the flatworm (*Dugesia tigrina*) model (Serhan et al., 2012). In a rabbit periodontitis model, RvE1 showed the ability of regenerating lost alveolar bone following three weeks of treatment (Hasturk et al., 2007). Further experiments explain the possible mechanisms of alveolar bone regeneration induced by SPMs. In vitro, RvE1 inhibits osteoclast differentiation and fusion, and restores osteoprotegerin (OPG) production of bone cells in the inflammatory condition (Herrera et al., 2008, Gao et al., 2013, Zhu et al., 2013). SPMs not only have an impact on bone cells, but also on cells of gingival tissue. RvD1 significantly enhances proliferation, wound closure, and fibroblast growth factor (FGF) release of human periodontal ligament (PDL) cells. Also, RvD1 reduces cytokine-induced prostaglandin E2 production and increases lipoxin production of human PDL cells (Mustafa et al., 2013).

In addition to previously mentioned disadvantages, taking NSAIDs impedes the resolution phase because they block the production of prostaglandins, which enhance the synthesis of SPMs (Levy et al., 2001, Serhan et al., 2007, Chan and Moore, 2010).

Therefore, SPMs, which are endogenous autacoids, can replace NSAIDs in treating inflammation by not only inhibiting inflammation but also actively inducing and enhancing the resolution phase. Given periodontitis is a multispecies biofilm-induced inflammatory disease, SPMs application could be a promising approach to treat periodontitis. The ability of SPMs (e.g. RvE1) in preserving and regenerating periodontal tissue in experimental periodontitis has been demonstrated (Hasturk et al., 2006, Hasturk et al., 2007). In addition to animal and in vitro studies, clinical evidence also supports the effect of SPMs on treating periodontitis. The ratios of precursors of pro-resolution/ pro-inflammatory lipid mediators were higher in healthy subjects than the ratios in aggressive periodontitis subjects (Elabdeen et al., 2013), suggesting that the lack of SPMs might be associated with periodontitis. In one clinical trial, adjunctive daily omega-3 fatty acid dietary supplement (900mg EPA+ DHA) with aspirin 81mg improved the clinical outcomes of scaling and root planing significantly three and six months after therapy. The percentage of probing depth >4mm significantly changed from 59.6% to 20.5% in the experimental group compared to the change in the control group (from 55.4% to 45.3%). Moreover, the salivary receptor activator of nuclear factor kappa- β ligand (RANKL) and matrix metalloproteinase-8 (MMP-8) levels showed significant reductions compared to the levels in subjects having scaling and root planing alone (El-Sharkawy et al., 2010). All these results support the possibility of applying SPMs for treating periodontitis, and RvE1 (Fig. 1.2) seems to have the strongest potential based on the available evidence.

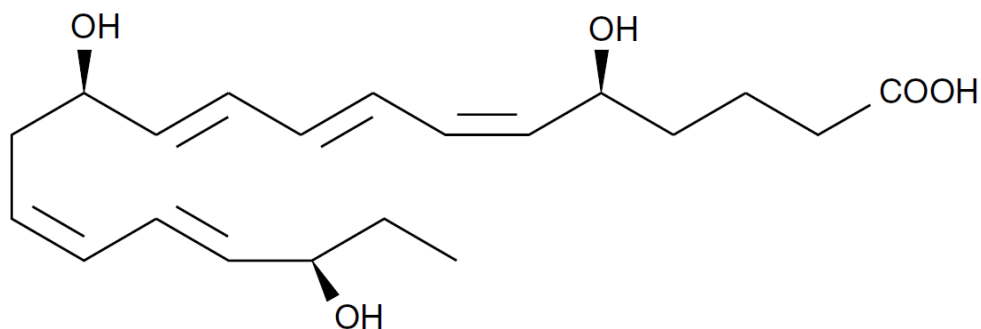


Figure 1.2 Structure of resolvin E1 (RvE1, $C_{20}H_{30}O_5$, molecular weight: 350.5)

RvE1 derived from EPA is one of the specialized pro-resolving mediators. It has been used to treat periodontitis (Hasturk et al., 2006, Hasturk et al., 2007), peritonitis (Arita et al., 2005b, Arita et al., 2005a, Bannenberg et al., 2005, Schwab et al., 2007), allergic airway inflammation (Haworth et al., 2008), heart reperfusion injury (Keyes et al., 2010), and retinopathy (Connor et al., 2007) in different animal models. The function of RvE1 in promoting inflammation resolution have been demonstrated from various perspectives, including stopping transepithelial and transendothelial migration of neutrophils, stimulating macrophages perform non-phlogistic phagocytosis of apoptotic neutrophils, blocking interleukin-12 (IL-12) production of dendritic cells, upregulating C-C chemokine receptor type 5 (CCR5) expression of apoptotic neutrophils and T cells (Serhan et al., 2008), inhibiting production of interleukin-4 (IL-4) and interferon-gamma (IFN- γ) of activated CD4 T cells in skin lesion model (Kim et al., 2012), enhancing phagocytosis and ROS production of neutrophils (El Kebir et al., 2012), rescuing phagocytosis of macrophages of localized aggressive periodontitis patients (Fredman et al., 2011), and inhibiting myeloperoxidase suppressed neutrophil apoptosis (El Kebir and Filep, 2013). These reactions induce the resolution phase and help tissue environment

reach homeostasis. Moreover, as previously mentioned, RvE1 regulates bone remodeling and improves bone regeneration (Gao et al., 2013, Zhu et al., 2013). RvE1 serves to regenerate destructive tissues following activating inflammation resolution.

The Temporal Relationship Between Biofilm Dysbiosis and Inflammation

As for the application of SPMs in treating periodontitis or other bacteria-induced inflammatory diseases, the impact of SPMs on the microbiota has to be discussed. In the rabbit *P.gingivalis*-induced periodontitis model, *P.gingivalis* (10^9 CFU) was inoculated around the ligated teeth in the first six weeks of disease induction phase. The inoculation was discontinued in the subsequent six-week treatment phase. At the end of six-week RvE1 application during the treatment phase, regeneration of lost alveolar bone was accompanied by elimination of *P.gingivalis*. Cell counts of some bacterial species, including *Prevotella intermedia*, *Fusobacterium nucleatum*, *Streptococcus intermedius*, significantly increased from baseline to the end of treatment (Hasturk et al., 2007). The detailed dynamics of microbial changes and the mechanism resulting in these changes were not determined. Two hypotheses have been proposed to explain these findings : (i) resolvins might have promoted the release of antimicrobial peptides, such as defensins and other bactericidal/permeability-increasing proteins, increased phagocytosis activity, stimulated nitric oxide synthase (NOS), or production of ROS in immune cells; or (ii) the resolution of the inflammation “starved” *P. gingivalis* because this species depends on peptides derived from host-tissue degradation as a source of nutrients, such as hemin (Van Dyke, 2008, Van Dyke, 2011). Several studies have shown increased killing ability of immune cells stimulated by SPMs. RvD2 increased neutrophil phagocytosis and ROS

production in *E.coli* phagocytosis assay and also reduced colony-forming units (CFU) by stimulating macrophage phagocytosis in a caecal ligation model (Spite et al., 2009). RvD1, RvD5 and PD1 enhanced phagocytosis of neutrophil and macrophage, and stimulated ROS production by neutrophils. Combination of SPMs (e.g. RvD1, RvD5, or PD1) and antibiotics reduced the bacterial load in the mice peritonitis model and the mice skin pouch model (Chiang et al., 2012). RvE1 enhanced phagocytosis of human PMN in vitro. Neutrophil phagocytosis evoked a rapid, robust ROS production and increased caspase-3,8 activity in murine acute lung injury model following RvE1 application (El Kebir et al., 2012, El Kebir and Filep, 2013). In summary, resolvins regulate phagocytosis and ROS production in the neutrophils and macrophages to kill bacteria. No appreciable direct antimicrobial effect of SPMs has been observed. These mechanisms could actively cause a change in the composition of the subgingival microbiota. However, the shift in microbial composition might still be indirectly caused by the resolution of the inflammatory environment. Further experiments need to be conducted to elucidate these mechanisms.

Animal Models of Experimental Periodontitis

To select an appropriate animal model to study the question of interest is a crucial part of the research project. There are several animal models that have been used to study the microbiota, immune response, and treatment effect in periodontal diseases. Different animal models have advantages and disadvantages, and no single animal model is perfect.

The first thing to consider when selecting the animal model is which animal species should be used. Non-human primates, dogs, miniature pigs, rabbits, ferrets,

hamsters, mice, rats and others have been used to study periodontal disease. Non-human primates, such as monkeys, are assumed to be the best species used to study human disease since their anatomic structures and physiological systems are much closer to humans than are other species. Some of the non-human primates, including rhesus monkeys (*Macaca mulatta*), cynomolgus monkeys (*Macaca fascicularis*), and baboons (*Papio anubis*), have naturally occurring dental plaque, calculus, human oral bacterial species (e.g., *Prevotella*, *Porphyromonas* species), and periodontal disease (Schou et al., 1993). Non-primate model established the fundamental knowledge of pathogenesis of periodontitis decades ago (Page and Schroeder, 1976). In order to expedite the induction of periodontal disease, ligatures or orthodontic wires can be placed around teeth (Kennedy and Polson, 1973, Kantor, 1980). These ligatures or wires serve as plaque traps and stimulants to induce immune responses resulting in periodontal disease. Nowadays, it is very difficult to obtain approval for using non-human primates in research if there is no undeniable reason. Due to stricter ethical rules and the necessary expense and special facility, non-primates models rarely have been used to study periodontal disease.

Miniature pigs have naturally occurring periodontal disease and human oral pathogens, such as *P. gingivalis* (Wang et al., 2007). Periodontal disease can also be induced or progressed by placing bands or wires between teeth (Lang et al., 1998, Van Dyke et al., 2015). Miniature pigs are relatively more expensive than rodents which are widely used in scientific research, and not many studies of periodontal disease using miniature pigs are available (Oz and Puleo, 2011).

The pathogenesis of periodontal disease in dogs has been studied for decades (Lindhe et al., 1973). Canine models are frequently used in the studies of periodontology and implantology because it is relatively easy to conduct periodontal or implant surgery in dogs compared to smaller animals (Berglundh et al., 1991, Wikesjo et al., 2003). Also, periodontitis can be induced in several weeks by placing the ligature around the tooth or changing diet to allow plaque accumulation (Lindhe et al., 1992). Periodontitis does occur naturally (Haney et al., 1995) and progresses with age in dogs. Several periodontal pathogens in humans were detected in dogs (Rober et al., 2008). However, the relatively high costs limit the popularity of dog models.

The rabbit model is usually used to perform surgery. The tibia or fibular of rabbits is usually used to place implants or regeneration materials (Johnson et al., 1997, Schmitt et al., 1997). In the periodontal abscesses of rabbits, several human periodontal bacteria, such as *Fusobacterium nucleatum*, *Peptostreptococcus micra*, *Actinomyces israelii*, can be isolated (Tyrrell et al., 2002). Periodontitis can be induced by *P. gingivalis* on the ligatures placed around teeth (Hasturk et al., 2007). The roots of teeth in rabbits continually grow and rabbits' teeth have different shapes from human teeth. Not many studies of experimental periodontitis in rabbits are available.

Compared to other larger animals, rodents are more cost-effective for scientific research. The expense of purchasing and raising rodents is much less than is the expense of purchasing and raising larger animals. It is also relatively easy to handle rodents because of the small body size. Mice and rats are the most frequently used rodents as an animal model in periodontal disease and other scientific projects. The immune systems

and anatomic structures in mice and rats are well-studied. In general, mice and rats have very similar physiological systems and genomes (Gibbs et al., 2004, Brudno et al., 2004). In the past 20 years, the application of transgenic murines, especially mice, makes murine models even more widely used.

Given periodontitis is induced by an imbalanced immune response, it is important to know the characteristics of immune systems in mice and rats. Mice are the mainstay of *in vivo* immunological experimentation because many biological mechanism in humans can be observed in mice (Mestas and Hughes, 2004). More than 90% of the mouse and human genomes can be partitioned into corresponding regions of conserved synteny and only about 300 genes appear to be unique to one species, reflecting the conservation of function in both species. (Mouse Genome Sequencing et al., 2002). However, there are still several key differences of the immunological systems in humans and mice (Shay et al., 2013). The proportions of leukocytes are quite different in the two species. Human blood is neutrophil rich (50–70% neutrophils, 30–50% lymphocytes) whereas mouse blood has a preponderance of lymphocytes (75–90% lymphocytes, 10–25% neutrophils) (Doeing et al., 2003). Some featured characteristics of leukocytes and immunity are also different. In humans, neutrophils are a rich source of leukocyte defensins, but defensins are not expressed by neutrophils in mice (Risso, 2000). IFN- α promotes Th1 cell differentiation in humans, but not in mice (Farrar et al., 2000). Some cytokines and receptors, such as interleukin-8 (IL-8), neutrophil-activating protein-2 (NAP-2 or CXCL7), interferon-inducible T-cell alpha chemoattractant (ITAC or CXCL11), chemokine receptor 1 (CXCR1), are missing in mice (Olson and Ley, 2002, Zlotnik and

Yoshie, 2000). Although the murine model is the most popular animal model, the distinct physiological systems between murines and humans should always be kept in mind.

Regarding the periodontitis model, the complexities of the subgingival microbiota and immune response in humans cannot be closely duplicated in any in vitro environment (Graves et al., 2008). However, it is known that the pathogenesis of experimental periodontitis in mice and rats is close to humans (Roy C. Page, 1982, Klausen, 1991, Fine, 2009). Oral bacteria induce immune response starting with the infiltration of neutrophils and recruitment of macrophages, and then the lymphocytes invade the inflamed periodontium. The disease progresses with epithelium elongation, degradation of tissue fibers, and resorption of alveolar bone. Various models have been utilized to study periodontitis in mice and rats, such as the air pouch model, (Gilroy et al., 1998), the chamber model (Genco et al., 1991, Gyurko et al., 2003), the calvaria model (Zubery et al., 1998), the ligature model (Rovin et al., 1966), the gavage model (Baker et al., 1994), the feeding model (Fine et al., 2001), and the lipopolysaccharide injection model (Dumitrescu et al., 2004). The air pouch is produced by injecting sterile air subcutaneously into the back of a rat or a mouse. The chamber model is produced by surgically implanting a chamber made of stainless-steel wire in the tissue of the dorsolumbar region. The bacterial or inflammatory stimulants, such as lipopolysaccharide, can be injected into the pouch or chamber, and the fluid or tissue samples can be collected following a specific period of inflammation. The air pouch model and chamber model are designed to investigate the interaction between periodontal pathogen(s) or inflammatory irritant(s) and immune cells in a localized environment. The calvarial

model has been adapted to study the effect of bacteria on bone resorption or the host–bacteria interactions on connective tissues (Graves et al., 2008). A stimulus is injected directly into the connective tissue overlying the calvarial bone to induce an inflammatory reaction. The production of pro-inflammatory cytokines and the recruitment of neutrophils can be observed in hours (Graves et al., 2005), and bone resorption can be induced within five days (Li et al., 2002). In the air pouch model, chamber model, and calvaria model, no infection or inflammation is established in periodontal tissues. The goal of these models is to study the inflammatory reaction in a well-controlled environment by injecting a known stimulus.

In order to observe the clinical changes, such as alveolar bone resorption, collagen fiber degeneration and epithelium migration, it is necessary to induce periodontal disease around teeth. Moreover, the interaction between host and microbiota around the periodontium is still much more realistic than the interaction happening in other tissues. Periodontitis does occur naturally in mice and rats, but it takes more than one year to be observed (Liang et al., 2010). Therefore, injection of lipopolysaccharide, ligature placement, and inoculation of human oral pathogen are often used to accelerate disease progression.

Lipopolysaccharide injection model

Lipopolysaccharide (LPS), which is a component of the cell wall of the gram negative bacteria, is a strong inflammatory stimulus triggering innate immunity. The aim of LPS injection model is to examine the innate immune response and the change of periodontal tissue following the stimulation of LPS. This model produces a

histopathological change similar to that observed in human periodontitis, characterized by increased infiltration of leukocytes, enhanced levels of pro-inflammatory cytokines, collagen degradation and alveolar bone resorption. Significant destruction of periodontal tissue can be observed within three to eight weeks in mice (Sartori et al., 2009) or rats (Rogers et al., 2007). Compared to the gavage model and ligature model, the inflammation in periodontal tissue is more controllable by injecting definite amount of LPS.

Oral gavage model

Several bacterial species associated with periodontitis in humans have been used in the oral gavage model, including *P. gingivalis* (Lalla et al., 1998), *A. actinomycetemcomitans* (Garlet et al., 2006), *T. forsythia* (Sharma et al., 2005) and *Treponema denticola* (Lee et al., 2009). Inoculation of these species can induce alveolar bone loss both in mice and rats. Different strains of animals have different degrees of reactions due to genetic variation (Breivik et al., 2001, Nakamura et al., 2008, Hiyari et al., 2015).

Typically, a specific amount of bacteria (10^9 CFU) mixed in the suspension (2% carboxymethylcellulose) is inoculated in the oral cavity of the animal. Bacteria usually only stay in the oral cavity transiently, and do not colonize permanently. Different bacterial species persist for different periods following the inoculation. In a rat model, *P. gingivalis* has been detected in the oral cavity for up to 11 weeks (Bainbridge et al., 2010). The periodontitis associated bacteria, including *P. gingivalis*, *T. denticola* or *T. forsythia*, can be inoculated separately or together to induce disease, and are detected four to six

weeks following the inoculation. These bacteria exhibit synergistic virulence resulting in significant inflammation-induced bone resorption (Kesavalu et al., 2007). In addition to the direct stimulation by the exogenous bacteria, the inoculation of periodontal pathogens, such as *P.gingivalis*, can cause a change of the original oral microbiota by manipulating the immune response to induce disease (Hajishengallis et al., 2011).

In the gavage model, the inoculation of bacteria usually continues for one to two weeks and significant alveolar bone loss can be observed from two weeks (Okada et al., 2010) to four weeks (Baker et al., 2000, Yu et al., 2007) following the last inoculation. This model induces an inflammatory reaction similar to periodontitis in humans, such as the infiltration of neutrophils and monocytes in the gingival connective tissue, increased inflammatory cytokines (TNF- α , IL- 12 and IFN- γ) and decreased anti-inflammatory cytokines (IL-10), proliferation of the junctional epithelium, and increased levels of serum antibody (IgG1 and IgG2) against the periodontal pathogens (Bainbridge et al., 2010, Lee et al., 2009, Garlet et al., 2005). The advantage of this model is having human oral pathogens to induce periodontitis. However, the oral gavage model usually takes at least four weeks to observe the initial alveolar bone loss.

Feeding model

The idea of inoculating human periodontal disease related bacteria in animal is to investigate the specific species simulating the periodontal infection. However, many of the human oral pathogens are not able to colonize in animal's oral cavity for long periods of time since the animals do not harbor these bacteria naturally. The species, *A. actinomycetemcomitans*, is a periodontal pathogen observed in different types of animals,

including humans, monkeys, and rats (Fine et al., 2005). *A. actinomycetemcomitans* is highly associated with localized aggressive periodontitis (Fine et al., 2007). In the feeding model, rats are pretreated with antibiotics and fed *A.actinomycetemcomitans* in their diets for four days. The pathogen, *A.actinomycetemcomitans*, could be observed in the mucosa or saliva weeks or months after the last feeding (Fine et al., 2001). However, only a specific strain of *A.actinomycetemcomitans* (CU1010) can induce periodontitis successfully in animals (Fine et al., 2001). Rats usually are used in the feeding model because it is difficult to colonize the oral cavity of mice with *A.actinomycetemcomitans* (Graves et al., 2012).

In addition to innate immunity, adaptive immunity is also induced in the feeding model. B cells and CD4 T cells are activated and their numbers increase, resulting in enhanced isotype-switched serum IgG, increased level of several cytokines and proteins, including IL- 1, IL- 2, TNF, CD40 ligand, Fas ligand (FasL), RANKL and osteoprotegerin in the lymph nodes (Li et al., 2010).

The exclusive use of *A.actinomycetemcomitans* in the feeding model limits the popularity of this model given that *A.actinomycetemcomitans* is generally recognized as a putative pathogen for localized aggressive periodontitis, but not necessarily highly related to chronic periodontitis.

Ligature Model

Ligature placement around teeth to induce periodontal disease is widely used in different species of animals, such as monkeys, dogs, mice, and rats (Struillou et al., 2010,

Oz and Puleo, 2011). The amount of bacteria increases significantly around the area of ligature placement given that the ligature traps plaque (Duarte et al., 2010). Presence of bacteria is required to induce periodontitis in this model. Germ-free rats do not have alveolar bone loss after ligature placement (Rovin et al., 1966). Treatment of chlorhexidine or antibiotics significantly inhibits the loss of alveolar bone in the ligature model (Weiner et al., 1979, Kenworthy and Baverel, 1981). In addition to accumulating bacteria, ligature causes traumatic wound which establishes an inflammatory environment for oral pathogens to grow. For example, the pathogen, *Eikenella corrodens*, does not cause alveolar bone loss in rats without ligature placement (Samejima et al., 1990). The commensal bacteria become primed to induce periodontal disease in mice following ligature placement (Jiao et al., 2013).

The ligature model can induce alveolar bone loss in one to two weeks which is a shorter period of time compared to other models (Bezerra et al., 2000, de Lima et al., 2000, Benatti et al., 2003). Some studies report the period of disease induction of up to two months (Kenworthy and Baverel, 1981, Holzhausen et al., 2002, Kuhr et al., 2004, Nakamura-Kiyama et al., 2014). The varied period of disease induction depends on the study design and characteristics of the ligature (e.g. diameter of ligature). Generally, in ligature models, the most significant alveolar bone loss happens within two weeks and then bone destruction starts to slow down (Kuhr et al., 2004).

Similar to the initial phase of periodontitis in humans, increased vascular permeability and leukocytes infiltration are observed following one week of ligature placement (Gyorfi et al., 1994). Some studies show inhibition of inflammatory cytokine

(Bezerra et al., 2000) or matrix metalloproteinase (Bezerra et al., 2002, Cesar Neto et al., 2004) controls the progress of periodontitis in the ligature model. These results mean the disease progression is accompanied with locally or systematically increased levels of inflammatory cytokines, such as IL-1, IL-6, and TNF- α (Endo et al., 2010, Gaspersic et al., 2003). The periodontitis related risk factors, such as smoking (Cesar Neto et al., 2004) and diabetes (Liu et al., 2006), also affect the loss of alveolar bone in the ligature model. This experimental periodontitis model simulates the progression of periodontitis in humans to a certain extent.

Compared to the oral gavage model, which initiates experimental periodontitis by inoculating high doses of human periodontal pathogens around animal teeth, the ligature model has the advantage of representing the natural process of biofilm-induced inflammation. The reasons are that periodontal pathogens usually only have relatively low abundance in human periodontal microbiota and these pathogens are present in the oral cavity even before the subjects have periodontal disease (Teles et al., 2013). In the ligature model, excess bacteria accumulate around the ligature to initiate the experimental periodontitis (Abe and Hajishengallis, 2013). For humans, total cell counts of bacteria and total mass of plaque are usually positively related to the severity of chronic periodontitis. Moreover, mice and rats do have some oral pathogens, which are similar to human periodontal pathogens, detected in the ligature model (Duarte et al., 2010, Jiao et al., 2013). Taken together, the ligature-induced periodontitis model might be a relatively more appropriate model to study microbiota in experimental periodontitis than the oral gavage model. Also, it is practical to collect plaque samples from the ligatures. However,

it is still debatable if the periodontal tissue inflammation initiated by the trauma of ligature placement does completely represent the inflammation environment of periodontitis in humans.

Subgingival Microbiota in Periodontitis: Biased vs. Unbiased Analytical Approaches

Bacteria-mediated inflammation is the main etiology of periodontitis. Oral biofilm attached on the root surface is the habitat for the periodontal microbiota. Different microbiotas form in oral mucosa, tongue, gingiva, and the tooth surface. The composition of the microbiota reflects its specific habitat in the oral cavity as well as changes in the local environment that is induced by intrinsic metabolism of the microbiota (Mager et al., 2003, Kolenbrander et al., 2010). In the microbial community, each species is colonized sequentially and has reciprocal interaction with others. A spatiotemporal model of periodontal microbiota colonization has been proposed (Kolenbrander and London, 1993).

The initial colonizers, such as *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguinis*, bind to complementary salivary receptors, including sialylated mucins, proline-rich protein, α -amylase, salivary agglutinin and bacterial cell fragments, in the pellicle coating the crown or root surface. Then the early colonizers, such as *Actinomyces naeslundii*, *Capnocytophaga gingivalis*, *Capnocytophaga ochracea*, and late colonizers, such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, bind previously bound bacteria sequentially. In the microbial complex, many species coaggregate with each other to stabilize the network. For example, *Fusobacterium spp.* plays an important role in bridging early and late colonizers. Different species also

change genetic information or provide nutrients to each other to survive in the community.

In 1998, Dr. Sigmund Socransky proposed the concept of periodontal microbial complexes based on the results of multiple cluster and community ordination analyses from more than 13,000 plaque samples. The complexes are named by different colors, and the species in the same complex are associated with each other temporally and spatially (Socransky et al., 1998). Moreover, increased total cell counts of species in specific complexes (e.g. orange or red complexes) and increased relative proportions of specific complexes are associated with the status of the periodontal tissues. The concept of periodontal microbial complexes has been widely used while analyzing human plaque sample.

Tooth-associated oral biofilms can be roughly divided into supragingival biofilms (on exposed tooth surface or above the gingival margin) and subgingival biofilms (below the gingival margin and within the periodontal pocket or sulcus) (Kolenbrander et al., 2010). The microbial community living in the biofilm is categorized into supragingival microbiota or subgingival microbiota. The supragingival microbiota not only plays an important role in caries formation, but also initiates the formation of subgingival microbiota. The supragingival microbiota, as detected by checkerboard DNA-DNA hybridization, is reestablished within two days following the cleaning of supragingival plaque (Haffajee et al., 2008). The species in the purple (e.g. *Veillonella parvula*), green (e.g. *C. gingivalis*, *E. corrodens*), and orange complexes (e.g. *F. nucleatum*) flourish during seven days of new biofilm formation. The sequences of bacterial colonization are

similar to the sequences proposed in the spatiotemporal model of periodontal bacterial colonization. The composition of supragingival microbiota and total cell counts of bacterial species between the healthy subjects and the periodontitis subjects are slightly different during the reformation of oral biofilms (Haffajee et al., 2008).

Having a similar design to Haffajee et al. (2008), another study investigates the change of subgingival microbiota during one week of biofilm reformation (Uzel et al., 2011). Unlike the supragingival microbiota, the pattern of subgingival microbiota reformation between the healthy subjects and the periodontitis subjects is significantly different. The cell counts of bacterial species detected by checkerboard DNA-DNA hybridization in the periodontitis subjects increase more rapidly than do these in the healthy subjects. The difference might be explained by a larger source of nutrients provided by the elevated flow of gingival crevicular fluid and many residual cells in the diseased periodontal pockets that contribute to the repopulation of the microbiota. The results of these two studies (Haffajee et al., 2008, Uzel et al., 2011) indicate distinct environments affecting the composition of the local microbiota. Although the microbial composition of supragingival plaque is associated with subgingival microbiota and periodontal status clinically to a certain degree (Mayanagi et al., 2004, Haffajee et al., 2008), the supragingival microbiota cannot completely represent the subgingival microbiota, especially in a diseased site with deep probing depth (Teles et al., 2006). Therefore, most studies collect subgingival plaque to evaluate the association between microbial composition and clinical status in periodontitis patients.

The interaction between the microbial and non-microbial components (e.g. immune cells, nutrients) of an ecosystem ultimately leads to homeostasis in which microbial and non-microbial forms exist in harmony and equilibrium with their environment. This is the climax community (Socransky and Haffajee, 2005). Specific climax communities exist in distinct environments. Therefore, the subgingival microbiota in healthy subjects and periodontitis subjects are very different given that the periodontal environment is distinct. When a tooth has periodontitis, the depth of the gingival sulcus increases due to the loss of periodontal attachment and alveolar bone. The inflammation happening in the periodontal tissue and the deepened gingival sulcus causes the changes of metabolism resulting in increased pH, increased variety of nutrients, elevated flow of gingival crevicular fluid, elevated temperature, decreased oxygen level, and low reduction potential. The changed environmental conditions favor the growth of periodontal pathogens, such as *P. intermedia*, *P.gingivais*, *T.denticola*, which have generally low numbers in the healthy periodontal environment (Marsh and Devine, 2011). Moreover, the gene expressions of the species also change in the periodontitis environment. The upregulation of putative virulence factor could enhance inflammation (Duran-Pinedo et al., 2014). The change of environment affects the microbiota, and changes in the microbiota also affect the environment.

There are many methods to investigate the subgingival microbiota. Culture method is the traditional way to identify bacteria, but this method cannot identify large numbers of bacterial species at the same time. Moreover, the inability of identifying uncultivable bacteria is a major issue to analyze complex microbiotas (e.g. microbiota in

the gut and the oral cavity). Therefore, the use of molecular approaches became the preferred method for studying the microbiota of different habitats nowadays. In periodontal research, several culture independent techniques have been frequently utilized: quantitative real-time PCR (qPCR), checkerboard DNA-DNA hybridization, and microarray hybridization (16S ribosomal RNA-based) (Teles et al., 2013). qPCR gives the relative amount of bacterial species after amplification, but the number of species analyzed in each sample at the same time is limited (Sakamoto et al., 2001).

Checkerboard DNA-DNA hybridization can detect multiple species simultaneously and gives the absolute counts of bacterial species, but each membrane only quantifies a maximum of forty-five cultivable species in a specified range (the DNA amount of a single species in a given sample should range between 10^4 to 10^7) (Socransky et al., 1994, Socransky et al., 2004). Microarray hybridization gives the relative detected frequencies of multiple species simultaneously (over 300 species), but the frequency of each species can be biased by the 16S rRNA gene PCR amplification process (Paster et al., 2006).

Next-generation sequencing (NGS) is an open-ended molecular technique, which is different from the other closed-ended methods mentioned above. The detection of bacteria in the microbiota is not restricted by the specificity of the probes or primers as in other techniques (Griffen et al., 2012). Large numbers of known and unknown bacterial species can be detected in an environmental or clinical sample by next-generation sequencing through “metagenomics” (Thomas et al., 2012). Metagenomic studies are commonly performed by analyzing the specific regions of prokaryotic 16S ribosomal RNA gene (16S rRNA) to conduct phylogenetic classifications such as genus or species

in diverse microbiota. 16s rRNA is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. The difference of copy numbers of 16s rRNA in species may cause bias in the relative abundance results of NGS (Vetrovsky and Baldrian, 2013). More importantly, NGS can analyze the functions of species efficiently by RNA sequencing (RNA-Seq), exome sequencing, and chromatin immunoprecipitation sequencing (ChIP-Seq). Large numbers of gene sequences can be analyzed in a short period of time.

However, while conducting phylogenetic classifications, NGS can only generate relative abundance of different taxa, and the detected frequencies are biased during the PCR amplification step. Since different techniques have advantages and disadvantages, the technique which can best answer the experimental question should be utilized.

CHAPTER TWO: SUBGINGIVAL MICROBIOTA SHIFTS IN PERIODONTITIS FOLLOWING RESOLVIN E1 APPLICATION

Introduction

Periodontitis is a biofilm-induced inflammatory disease. The initiation and progression of periodontal disease requires the simultaneous occurrence of a number of factors, including the virulent periodontal pathogen, host susceptibility, and the local environment favoring expression of virulence factors (Haffajee and Socransky, 1994). Although it is still debatable whether bacteria or immune response or both are the primary initiators of periodontal disease progression, it is clear that the inflammatory response followed by the acquired immune response drives the pathogenesis of periodontitis after the initial bacteria challenge. Therefore, regulating the immune response appears to be a promising approach to treat periodontitis. Several years ago, the discovery of the active resolution phase of inflammation provided a missing link in our understanding of chronic inflammatory diseases. Chronic inflammation may be due to a failure of resolution, rather than too much inflammatory stimulation. If the resolution phase can be activated, the inflamed tissue will return to homeostasis and regeneration of tissue will occur (Serhan et al., 2008, Van Dyke, 2014). Specialized pro-resolving mediators (SPMs), including lipoxins, resolvins, protectins, maresins, are molecules which initiate the resolution phase. As discussed above, resolvins have already been used to treat periodontitis in animal models.

Previous studies demonstrated that the local application of RvE1 in a *P. gingivalis*-induced periodontitis model in rabbits prevented the onset of periodontitis and

promoted the regeneration of the periodontium destroyed by the disease (Hasturk et al., 2006, Hasturk et al., 2007). An interesting outcome of one of these studies in rabbits was the apparent spontaneous disappearance of *P. gingivalis* following the regeneration of periodontal tissue treated by resolvin E1 application (Hasturk et al., 2007). No mechanical or antimicrobial therapies were used. The rabbit ligature model experiment was initiated with a six-week disease induction phase (*P. gingivalis* inoculation) followed by subsequent six-week treatment phase (RvE1 application) after *P. gingivalis* inoculation was stopped. Microbial dental plaque (pooled supragingival and subgingival biofilm) was sampled at baseline, at six and twelve weeks. The authors reported that infection with *P. gingivalis* altered the composition of the existing oral microbiota of the rabbits, resulting in the detection of previously undetected species, such as *A. actinomycetemcomitans* and *F. nucleatum*, and in the disappearance of *Campylobacter curvus* and *Campylobacter rectus*, among other changes at the end of the disease induction. Application of RvE1 resulted in a return of the microbiota to its baseline composition and the apparent elimination of *P. gingivalis*, whereas placebo treated animals maintained the complex pathogenic microbiota. Two potential mechanisms were proposed to explain these findings: (i) RvE1 might have stimulated the antibacterial activities of immune cells; or (ii) the resolution of the inflammation “starved” *P. gingivalis* reducing nutrients which were derived from host-tissue degradation (Van Dyke, 2008). The dynamics of microbiota shift after inflammation resolution and tissue regeneration following RvE1 application is still not clear.

The goal of our studies was to characterize the kinetics of changes in the subgingival microbiota in an experimental periodontitis model after treatment of the disease with resolvin E1, and to start to explore the mechanisms leading to these changes. A ligature-induced periodontitis model in the rat was utilized because, unlike the rabbit model, which requires exogenous addition of *P. gingivalis* to induce disease, rats develop disease presumably induced by their own microbiota. The goal was to fill a gap in knowledge regarding the impact of specialized pro-resolving lipid mediators (SPMs) on the composition of the subgingival microbiota during periodontitis progression.

The hypothesis was that resolvin E1 would result in the resolution of periodontal inflammation leading to changes in the local environment that would lead in a shift in the composition of the adjacent subgingival biofilm. To test this hypothesis, we proposed two specific aims:

Specific Aim 1 – To examine the changes in the rat subgingival microbiota during the induction and treatment phases of ligature-induced periodontitis using checkerboard DNA-DNA hybridization and next-generation sequencing.

We hypothesized that after ligature placement, the total mass of the subgingival biofilm would increase, and the microbiota would shift towards a higher proportion of periodontal disease associated pathogens during disease progression. Conversely, resolution of the periodontitis following RvE1 application would reverse those changes and reestablish a health-associated microbiota.

In pilot studies, checkerboard DNA-DNA hybridization was used to determine whether shifts could be detected after RvE1 treatment. This relatively inexpensive screening tool employed probes to human pathogens, so the applicability to rat associated microbiota is relative. Next-generation sequencing is a powerful tool to perform metagenomics for the characterization of the rat microbiota associated with health and disease. Once the potential impact of RvE1 was established, this technique was used to characterize microbial dysbiosis caused by ligature placement and the impact of RvE1 in reversing this dysbiosis.

Specific Aim 2 – To characterize the clinical and local inflammatory response changes in the periodontium of rats.

We hypothesized that in the presence of RvE1, bone regeneration would be observed with a reduction of the inflammatory infiltrate in the tissues at the histological level. Tissue expression of specific inflammatory gene pathways should also be inhibited.

Materials and Methods

Animals and experimental periodontitis model

This experiment was approved by Institutional Animal Care and Use Committee (IACUC) of the Forsyth Institute. Eighteen six-week old male Wistar rats (weight 180-200 g, Charles River Laboratories, New York, NY) were used. All animals were maintained in a controlled temperature (22⁰ C to 25⁰ C) and dark/light cycle (12/12 hours) facility. Rats received a standard laboratory chow diet and water *ad libitum*. Rats were sedated by isoflurane 2-3% before being anesthetized with ketamine (80mg/kg,

intraperitoneally) and xylazine (16 mg/kg, intraperitoneally). It was necessary to anesthetize the rats before performing all experimental procedures, including placing the ligature, checking the ligature position, collecting subgingival plaques, or applying RvE1. Eighteen rats were divided into four groups (no ligature: n=6, ligature alone: n=6, ligature + Vehicle: n=3, ligature + RvE1: n=3). 3–0 silk ligatures were placed subgingivally on the maxillary right and left second molars of each ligated rat on the first day of the experiment to induce periodontitis. The knots were tightened on the buccal side of the maxillary second molars to prevent being disturbed by the tongue. The experimental period lasted six weeks, including a three-week disease induction phase and the subsequent three-week treatment phase. The stability of the ligatures was checked every other day for every rat. RvE1 ($C_{20}H_{30}O_5$, molecular weight: 350.5, purity>97%, λ_{max} : 272 nm) was obtained from Cayman Chemical (Ann Arbor, MI). The RvE1 provided by Cayman was prepared by stereospecific total synthesis guided by the published structure described in the published reference (Arita et al., 2005a). Analytical and biological comparisons with endogenously derived RvE1 have confirmed its identity as matching the natural product.

Six rats in the non-ligature (healthy) group were sacrificed on the first day of the experiment to collect baseline tissue samples. At the end of the disease induction phase (21st day), the six rats in the ligature alone group were sacrificed to quantify the amount of disease induced. During the treatment phase, 1 μ g RvE1 (Cayman Chemical Co. Inc., Ann Arbor, MI) dissolved in 4 μ l of ethanol was applied to the ligated teeth of the rats in the RvE1 group (2 μ l on buccal or palatal side respectively), and 4 μ l of ethanol (vehicle

alone) was applied on the ligated teeth of the rats in the Vehicle group with a Hamilton syringe (25G needle, 5µl syringe). RvE1 (0.7mM) and vehicle were applied every other day during the treatment phase (weeks four to six). At the end of six weeks, the rats from both remaining groups were sacrificed. All rats were euthanized by CO₂ inhalation. The timeline of the experiment is summarized in Fig. 2.1.

The following samples were collected: subgingival plaque, gingival tissue, maxillary bone block, and serum. Subgingival plaque samples were collected on day 0, 8, 12, 16, 20, 24, 28, 32, 36, and 40. Other samples were collected immediately after sacrifice. The maxillae were split into two halves: one half was taken for the analyses of bone morphometry and gene expression. The palatal gingiva of the ligated tooth was incised and stored in 200µl of RNAlater (Sigma-Aldrich, St. Louis, MO) for the qRT-PCR assay. Then the remaining specimen was defleshed by beetles to obtain a clean bone block. The other half was processed for histomorphometric analysis. Blood was obtained using heart aspiration and centrifuged to collect serum. Serum was aliquoted and stored at -80 °C (eight aliquots of 100µl each) for future analysis.

Bacterial DNA extraction and amplification

Subgingival plaque samples were collected using the tip of a 2-Whiteside scaler (Hu-Friedy, Chicago, IL) from the palatal site of the maxillary second molars. Before placing the ligature on the maxillary second molars, a plaque sample was collected from four teeth (two maxillary second molars and two mandibular second molars) of each rat at baseline and then the samples were pooled in one collection tube. The sample was collected from one ligated tooth of each rat each time after the ligature placement. The

DNA of subgingival plaque was extracted using the MoYsis Basic (CaerusBio Inc., Dowingtown, PA) and QIAamp® mini kit (QIAGEN Inc., Valencia, CA). The MoYsis Basic kit was used to isolate the bacterial DNA from the sample to exclude DNA of mammalian cells. Mammalian cells were lysed under chaotropic conditions to release DNA. The released mammalian DNA was enzymatically degraded, and then the bacterial cells were lysed (Horz et al., 2008, Horz et al., 2010). After selectively removing mammalian DNA, the bacterial DNA was extracted with QIAamp® mini kit (QIAGEN Inc., Valencia, CA). Briefly, the isolated bacterial cells were mixed with lysozyme (20 mg/ml) and Proteinase K, and incubated at 56 °C for two hours to lyse the cell wall to release DNA. DNA was then isolated and eluted following the manufacturer's instructions.

The extracted bacterial DNA was amplified with the multiple displacement amplification kit (GenomiPhi™ V3 DNA amplification kit, GE Healthcare Bio-Sciences, Pittsburgh, PA). Multiple displacement amplification (MDA) enables the whole genomic amplification of DNA targets (Dean et al., 2002, Brito et al., 2007). The unique $\Phi 29$ DNA polymerase used in this technique is able to amplify DNA isothermally at 30 °C and gives the most complete coverage, avoid sequence effects, and results in unbiased amplification. The MDA technique can increase the bacterial DNA amount up to one thousand fold. In brief, 3 μ l of each of the DNA templates was added to 7 μ l pure water in 200 μ l microcentrifuge tubes, and then was mixed with 10 μ l 2X Denaturation Buffer. Templates in denaturing buffer were heat denatured at 95 °C for 3 minutes in the thermal cycler and cooled to 4 °C. Then the 20 μ l of denatured DNA template was added to each

Ready-To-Go GenomiPhi V3 cake in the well. The wells were sealed with the domed caps provided. The mixtures were kept on ice prior to incubation. The samples were incubated at 30 °C for 1.5 hours for DNA amplification, and then the samples were heated to 65 °C for 10 minutes to inactivate the Φ 29 DNA polymerase enzyme. The amplification reactions were stored at -20°C for future use. The concentration of amplified DNA samples was measured with fluorescent nucleic acid stain (Quant-iT™ PicoGreen® dsDNA Assay, Life Technologies, Grand Island, NY).

Checkerboard DNA-DNA hybridization

Forty human periodontal disease associated bacterial probes (Socransky et al., 2004) (Table 2.1) were selected to detect the bacterial DNA of rat subgingival plaque after multiple displacement amplification using checkerboard DNA-DNA hybridization. In brief, 8 μ l amplified bacterial DNA was added in 1ml Tris EDTA buffer (10 mm Tris-HCl, 1 mm ethylenediaminetetraacetic acid, pH 7.6). Immediately after, the solution with DNA was placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After fixation of the DNA to the membrane, the membrane was placed in Miniblotter 45 (Immunetics, Cambridge, MA), with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to forty subgingival species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency. The DNA probes were detected using antibody to digoxigenin, and conjugated with alkaline phosphatase and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL) and were read using a Typhoon Trio Variable Mode

Imager (GE Healthcare Bio-Sciences, Pittsburgh, PA), a computer-linked instrument that reads the intensity of the fluorescence signals resulting from the probe-target hybridization. Two lanes in each run contained standards at the concentration of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals were evaluated using the Phoretix Array (TotalLab Ltd, UK) and converted to absolute counts by comparison with the regression line determined from data from the standards on the same membrane. Failure to detect a signal was recorded as zero.

Next-generation sequencing

The microbial composition of the plaque samples (50ng DNA/10 μ l) were characterized by sequencing the hyper-variable V3 and V4 regions of the 16S rRNA gene using the Illumina MiSeq® platform. Paired-end sequencing allows the capture of the entire V3-V4 region by overlapping and merging the read pairs. Successfully merged reads were processed through the QIIME pipeline (Caporaso et al., 2010) filtering out low quality sequences using a quality score threshold of 20. Operation Taxonomic Units (OTUs) were created by clustering the merged quality filtered reads at a 97% identity threshold using sequences from a reference database (SILVA/Greengenes) (DeSantis et al., 2006, Quast et al., 2013) as a guide. All remaining sequences entered a *de novo* clustering step where they were clustered at the same 97% identity threshold without guidance from the reference database. Potential chimeric OTUs were removed through the use of UCHIME (Edgar et al., 2011) and the gold 16 database (Public domain version of UCHIME, version 4.1, http://drive5.com/uchime/uchime_download.html). Taxonomic

assignments to OTUs from phylum to genus level were completed through QIIME's use of the given reference database (SILVA/Greengenes) and the UCLUST algorithm (Edgar, 2010). Further analysis included alpha diversity (e.g. Shannon evenness), beta diversity (weighted UniFrac) (Lozupone et al., 2006), principal coordinate analysis (PCoA) and relative abundance profiles of the microbial community in the samples.

Relative abundance of taxa in each sample was defined as dividing the hit of each taxon by the total hits of all taxa in the sample. In ecology, diversity is usually thought of as being composed of richness (the number of species) and evenness (the relative abundance of species). Alpha diversity is defined as the richness and evenness within a habitat unit (a sample). Beta diversity is defined as the expression of diversity between habitats (samples).

In this study, richness was evaluated by the number of observed OTUs, and evenness was measured as Shannon evenness or non-parametric Shannon Index (Anne Chao, 2003). A higher value of the number of observed OTUs means more species existing within a sample. A high value of Shannon index or non-parametric Shannon evenness is representative of a diverse and equally distributed community and a lower value represents a less diverse community. Beta diversity was represented by weighted UniFrac distance. UniFrac distance is used to measure the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree. UniFrac distance can determine whether communities are significantly different. Weighted UniFrac distance accounts for the relative abundance of each taxon within the

communities. The data in this distance matrix was visualized with principal coordinates analysis (PCoA).

Morphometric analysis

The dissected maxilla bones were defleshed and stored in 0.3% hydrogen peroxide for 24 hours. Then the bones were dried completely before being stained. The alveolar bone loss of three maxillary molars was analyzed using a dissecting microscope. The dissected maxilla bones were stained with methylene blue and the images (at 0.63X10 times magnification) were taken under the dissecting microscope (Axio observer A1, ZEISS) using AxioVision 4.8 software. The area of exposed root surface was measured at the buccal and palatal sites of three maxillary molars. The distance between the alveolar bone margin and the cemento-enamel junction (CEJ) was measured at nine sites (mesial, middle, and distal sites of the first molar; interproximal site between the first molar and the second molar; mesial and distal sites of the second molar; interproximal site between the second molar and the third molar; mesial and distal sites of the third molar) of three maxillary molars at the buccal site and the palatal site. The direction of the distance line was parallel with the axis of the root (Fig. 2.2). All measurements were performed using computer software (ImageJ). The measurements of buccal and palatal sites were added for statistical analysis.

Histomorphometry

Half of the maxilla was immersed in 20 volumes of 10% formalin for 48 hours and rinsed with water for four hours. Then the tissue specimen was immersed in 20

volumes of 10% EDTA, which was replaced every 48 hours for two to three weeks. The completion of decalcification was confirmed by the chemical reaction of calcium oxalate precipitation. After the process of decalcification, the tissue specimens were rinsed for four to eight hours in running water, and soaked in 50% and 70% ethanol sequentially. The tissue specimens were immersed in 90% ethanol the day before embedding with paraffin. Serial mesio-distal sections (6 μ m) parallel to the long axis of the teeth were cut. Thin sections were either stained with H&E for light microscopy and identification of the cellular composition of inflammatory infiltrates, or stained with tartrate resistant acid phosphatase (TRAP) to examine osteoclastic activity.

Sections with similar anatomic positions were selected for quantitative measurement. Different sections were measured randomly to avoid bias. Specific areas of mesial and distal interproximal sites were selected for histological assessment. Each measurement had three different sections. The number of inflammatory cells, including neutrophils, lymphocytes and plasma cells, was counted in the area of connective tissue above the alveolar bone at 400x magnification. The total number of osteoclasts around the interproximal bone was counted. The area of interproximal bone was measured. Osteoclast density was defined as dividing the number of osteoclasts by the area of interproximal bone. All the measurements were performed using computer software (ImageJ). All measurements in each sample were the mean of the mesial side and the distal site from three sequential sections.

Quantitative reverse transcription polymerase chain reaction assay

Total RNA was extracted from gingival tissue with Trizol reagent (Invitrogen, Grand Island, NY) as per the manufacturer's protocol. Gingival tissue was homogenized by pestle homogenizer before adding Trizol reagent. The concentration and purity of RNA was estimated by the A_{260}/A_{280} ratio spectrophotometrically (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA). A total of 1 μ g RNA was converted to cDNA using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Grand Island, NY). The 20 μ l reaction mixtures were thoroughly mixed and assayed at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for five minutes in a thermal cycler (ABI 9700, Applied Biosystems).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using primers and TaqMan probes for *Cxcl1*, *Ptgs2*, *Nos2*, and labeled with FAM dye (Applied Bio-systems, TaqMan gene expression assays). β -actin (*Actb*) was utilized as an internal control and amplified using preformulated VIC-TAMRA-labeled TaqMan probes (Applied Biosystems, Endogenous Control). Quantification was performed in an automated thermal cycler (StepOnePlus™ System, Applied Biosystems). The reaction mixtures were kept at 50°C for two minutes (one cycle), 95°C for 20 seconds (one cycle), 95°C for one second and 60°C for 20 seconds (40 cycles). The results were analyzed through a software interface and spreadsheet for the calculation of relative expression ($2^{-\Delta\Delta C_T}$).

The gene *Cxcl1* (*GRO- α* , *CINC*, *CXCL1* in humans) controls the production of chemokine (C-X-C motif) ligand 1 (CXCL1), which is a chemokine expressed in endothelial cells, fibroblasts, neutrophils, monocytes and other cells. CXCL1 is highly

chemotactic for neutrophils. For rodents, there is no IL-8 gene expression; therefore, CXCL1 plays an important role in recruiting the neutrophils during acute inflammation.

The gene *Ptgs2* controls the production of prostaglandin-endoperoxide synthase 2 (PTGS2 or COX-2), which is an enzyme involving in the conversion of arachidonic acid to prostaglandin H2 and thromboxane A2. COX-2 is unexpressed under normal conditions in most cells, but has elevated levels during inflammation. The expression of *Ptgs2* is associated with bone resorption, platelet aggregation, vessel tone and other physiological functions.

The gene *Nos2* controls the expression of inducible nitric oxide synthase (iNOS). The production of iNOS is stimulated by lipopolysaccharide and cytokines, such as IL-1 β , TNF- α , and IFN- γ . Inducible nitric oxide synthase produces large quantities of nitric oxide (NO) upon stimulation react with superoxide (O₂⁻) leading to peroxynitrite (ONOO-) formation and cell toxicity.

Statistical Analysis

The comparisons between two groups were analyzed by unpaired or paired two-tailed Student's t-test. The comparisons between multiple groups were analyzed by one-way analysis of variance (one-way ANOVA) and post hoc analysis was performed (pairwise t-test). All values were expressed as mean \pm standard error of the mean. P-value <0.05 was considered statistically significant.

Results

Ligature placement induces experimental periodontitis

After three weeks of ligature placement, significant bone loss was observed in the ligature alone group (mean area of exposed root surface in three molars- no-ligature (healthy): $3.2 \pm 0.4 \text{ mm}^2$, ligature alone: $7.3 \pm 0.6 \text{ mm}^2$, $p < 0.01$; mean distance between CEJ and alveolar bone level- no ligature: $4.1 \pm 0.5 \text{ mm}$, ligature alone: $9.9 \pm 0.8 \text{ mm}$, $p < 0.01$) (Fig. 2.3). Histologically, the mean area of interproximal bone in the ligature alone group was also significantly smaller than the area in the no ligature group (no ligature: $0.35 \pm 0.03 \text{ mm}^2$, ligature alone: $0.18 \pm 0.03 \text{ mm}^2$, $p < 0.01$).

RvE1 treatment regenerates lost alveolar bone

Three weeks of RvE1 treatment significantly reversed alveolar bone loss induced by ligature placement compared to vehicle application (mean area of exposed root surface in molars - Vehicle: $8.0 \pm 0.4 \text{ mm}^2$, RvE1: $5.8 \pm 0.2 \text{ mm}^2$, $p < 0.01$; mean distance between CEJ and alveolar bone level - Vehicle: $11.4 \pm 0.5 \text{ mm}$, RvE1: $8.3 \pm 0.2 \text{ mm}$, $p < 0.01$) (Fig. 2.4). The alveolar bone level in the RvE1 group was higher than that in the ligature alone group (mean area of exposed root surface in molars - ligature alone: $7.3 \pm 0.2 \text{ mm}^2$, RvE1: $5.8 \pm 0.2 \text{ mm}^2$, $p < 0.01$; mean distance between CEJ and alveolar bone level - ligature alone: $9.9 \pm 0.3 \text{ mm}$, RvE1: $8.3 \pm 0.2 \text{ mm}$, $p = 0.03$) (Fig. 2.4). Histologically, regeneration of interproximal bone following RvE1 treatment was also observed (mean area of interproximal bone- Vehicle: $0.16 \pm 0.01 \text{ mm}^2$, RvE1: $0.22 \pm 0.02 \text{ mm}^2$) (Fig. 2.5).

RvE1 treatment reduces osteoclast activity and inhibits inflammation in experimental periodontitis

The mean count of inflammatory cells in the connective tissue in the RvE1 group was lower than the count in the ligature alone and Vehicle group but the difference was not statistically significant (Fig. 2.6). RvE1 treatment significantly inhibited osteoclast activity compared to vehicle application ($p < 0.05$). Also, the osteoclast density in the RvE1 group was lower than that in the ligature alone group ($p = 0.02$) (Fig. 2.7).

Expression of inflammation related genes was inhibited by RvE1 treatment. The relative gene expression levels ($2^{-\Delta\Delta CT}$) of *Cxcl1*, *Ptgs2*, *Nos2* in the RvE1 groups were lower than the other two groups, and RvE1 treatment significantly reduced relative gene expression level of *Cxcl1* compared to vehicle application ($p = 0.02$).

Subgingival microbiota shift during disease induction phase and treatment phase

Pilot experiments were performed with checkerboard DNA-DNA hybridization to identify subgingival microbiota and analyze the cell counts (CFU) of 40 species derived from human samples. Since DNA probes were made of human bacterial DNA, the terminology “like species” was employed to describe the detection of probe signal (Rober et al., 2008, Duarte et al., 2010). Mean cell counts of 33 out of 40 species identified by checkerboard DNA-DNA hybridization significantly increased from the baseline to the end of disease induction phase (Table 2.2). Only five species (*Capnocytophaga gingivalis*, *Prevotella intermedia*, *T. forsythia*, *Propionibacterium acnes*, *Prevotella melaninogenica*-like species) did not exhibit statistically significant differences. Nine species, *Streptococcus oralis*, *Veillonella parvula*, *Streptococcus mitis*, *Streptococcus gordonii*, *Streptococcus intermedia*, *Streptococcus sanguini*, *Actinomyces gerencseriae*, *Streptococcus constellatus*, and *Eubacterium saburreum*-like species, had

cell counts more than 2×10^5 on day 20. Total cell counts significantly increased from baseline to the end of disease induction phase ($p < 0.01$).

RvE1 treatment significantly reduced bacteria at the end of treatment phase (day 40). At the beginning of treatment phase (day 20), mean cell counts of species had no significant difference between the Vehicle group and the RvE1 group, except for one species: *Campylobacter showae*-like species. On day 40, mean cell counts of species in the RvE1 group were lower than mean cell counts of species in the Vehicle group, except two species: *Streptococcus mitis* and *Streptococcus oralis*-like species (Fig. 2.9). Mean cell counts of nineteen species (e.g. *T. forsythia*, *P. gingivalis*-like species) in the RvE1 group were significantly lower than these in the Vehicle group on day 40 (Table 2.3). Total cell counts were significantly reduced by RvE1 treatment compared to vehicle application.

The data was grouped and represented as the relative proportions of periodontal microbial complexes as defined by Socransky. The pattern of baseline (day 0) was quite different from the patterns of other time points after disease induction. Yellow and other complexes (27.55%, 22.32%) had higher relative proportions than did other complexes on day 0. The yellow complex became the only dominant microbial complex after disease was induced (Table 2.4). During the treatment phase, RvE1 treatment limited changes of the relative proportions of periodontal microbial complexes compared to vehicle application. Before treatment started (day 20), the relative proportions of microbial complexes in the Vehicle group were similar to those in the RvE1 group. At the end of treatment (day 40), the Vehicle group had a relatively lower proportion of yellow

complex and higher proportions of orange and red complexes than did RvE1 group (Yellow complex - Vehicle: 33.76 %, RvE1:40.41 %; Orange complex - Vehicle: 20.53%, RvE1: 16.92%; Red complex: Vehicle: 4.34%, 2.93%, Table 2.5).

Having established an impact of RvE1 on the microbiota with the checkerboard pilot, the definitive experimental analysis of the rat microbiota was performed using next-generation sequencing. There was a clear shift in the subgingival microbiota following ligature placement (Fig. 2.10). Considering the variation of relative abundance and limited sample size, a less conservative significant level ($p < 0.3$) was chosen to find the potential genera representing subgingival microbiota in different groups. Several genera changed significantly from baseline to the end of the disease induction phase. Two genera, *Streptococcus* and *Rothia*, had the largest changes in mean relative abundance during disease induction phase (Fig. 2.11). In the treatment phase, the subgingival microbiota shift was compared between the RvE1 and the Vehicle treatment groups. At the phylum level, *Firmicutes* were dominant in the RvE1 group during the treatment phase. *Actinobacteria* and *Firmicutes* in the Vehicle group had similar relative abundance at the end of treatment phase (Table 2.6). Four genera (*Enterococcus*, *Veillonella*, *Allobaculum*, *Lactobacillus*) demonstrated more significant difference ($p < 0.3$) between the RvE1 group and the Vehicle group on day 32 than did other genera. On day 40, 11 genera (*Coprococcus*, *Lactobacillus*, *Blautia*, *Sutterella*, *Veillonella*, *Streptococcus*, *Corynebacterium*, *Collinsella*, *Facklamia*, *Dorea*, *Aggregatibacter*) had more significant difference ($p < 0.3$) between the RvE1 group and the Vehicle group than did other genera (Fig. 2.12).

Regarding the diversity of subgingival microbial community, the evenness (Shannon evenness) appeared to gradually increase during the whole experiment. At the end of the experiment, the Shannon evenness in the Vehicle group was significantly lower than that in the RvE1 group (Fig. 2.13). Richness of microbial community did not appear to change significantly during the phase of disease induction and the phase of treatment. The number of observed OTUs in the RvE1 group was lower than that in the Vehicle group at the end of treatment phase without statistical significance (Fig. 2.13). The results indicate the richness of microbial community was reduced by RvE1 treatment, and that the species in the RvE1 group were more evenly distributed than in the Vehicle group suggesting that RvE1 reduces the number of species represented in the biofilm and that no single species or group of species has overgrown.

The graphs of principal coordinated analysis (weighted UniFrac distance) demonstrate a significant shift of subgingival microbiota between the baseline and the end of disease induction phase (Fig. 2.14). The variation of weighted UniFrac distance between samples on day 0 is larger than the variation between samples on day 20 (Fig. 2.15) indicating the diversity between samples became smaller following disease induction. The diversity of the subgingival microbiota between the RvE1 group and the Vehicle group separated more at the end of treatment phase than did the subgingival microbiota at the beginning of treatment phase (Fig. 2.14). Taken together with Richness and Evenness data, the results indicate that RvE1 treatment increased diversity toward that associated with a healthy periodontium.

Table 2.1 List of forty species identified in the checkerboard DNA-DNA hybridization by the order of periodontal microbial complexes

^aPeirodental Microbial Complexes	Bacterial Species
Actinomyces	<i>Actinomyces gerencseriae</i>
Actinomyces	<i>Actinomyces israelii</i>
Actinomyces	<i>Actinomyces naeslundii I</i>
Actinomyces	<i>Actinomyces viscosus</i>
Purple	<i>Actinomyces odontolyticus</i>
Purple	<i>Veillonella parvula</i>
Yellow	<i>Streptococcus gordonii</i>
Yellow	<i>Streptococcus intermedius</i>
Yellow	<i>Streptococcus mitis</i>
Yellow	<i>Streptococcus oralis</i>
Yellow	<i>Streptococcus sanguis</i>
Green	<i>Aggregatibacter actinomycetemcomitans</i>
Green	<i>Capnocytophaga gingivalis</i>
Green	<i>Capnocytophaga ochracea</i>
Green	<i>Capnocytophaga sputigena</i>
Green	<i>Eikenella corrodens</i>
Orange	<i>Campylobacter gracilis</i>
Orange	<i>Campylobacter rectus</i>
Orange	<i>Campylobacter showae</i>
Orange	<i>Eubacterium nodatum</i>
Orange	<i>Fusobacterium nucleatum ss nucleatum</i>
Orange	<i>Fusobacterium nucleatum ss polymorphum</i>
Orange	<i>Fusobacterium nucleatum ss vincentii</i>
Orange	<i>Fusobacterium periodonticum</i>
Orange	<i>Parvimonas micra</i>
Orange	<i>Prevotella intermedia</i>
Orange	<i>Prevotella nigrescens</i>
Orange	<i>Streptococcus constellatus</i>
Red	<i>Tannerella forsythia</i>
Red	<i>Porphyromonas gingivalis</i>
Red	<i>Treponema denticola</i>
Others	<i>Eubacterium saburreum</i>
Others	<i>Gemella morbillorum</i>
Others	<i>Leptotrichia buccalis</i>
Others	<i>Neisseria mucosa</i>
Others	<i>Propionibacterium acnes</i>
Others	<i>Prevotella melaninogenica</i>
Others	<i>Streptococcus anginosus</i>
Others	<i>Selenomonas noxia</i>
Others	<i>Treponema socranskii</i>

^aThe species are listed in complex order (Actinomyces, Purple, Yellow, Green, Orange, Red, Others)

Table 2.2 Mean counts ($\times 10^5$) (Mean \pm SEM) of 40 species in the disease induction phase

Visit		Day 0 ($\times 10^5$)	Day20 ($\times 10^5$)
Complex	Species		
Actinomyces	<i>Actinomyces gerencseriae</i>	0.95 \pm 0.19	2.31 \pm 0.15*
Actinomyces	<i>Actinomyces israelii</i>	0.38 \pm 0.08	0.75 \pm 0.10*
Actinomyces	<i>Actinomyces naeslundii I</i>	0.39 \pm 0.07	2.64 \pm 0.18*
Actinomyces	<i>Actinomyces viscosus</i>	0.45 \pm 0.09	1.05 \pm 0.07*
Purple	<i>Actinomyces odontolyticus</i>	0.77 \pm 0.16	1.67 \pm 0.13*
Purple	<i>Veillonella parvula</i>	0.49 \pm 0.07	11.50 \pm 0.23*
Yellow	<i>Streptococcus gordonii</i>	0.73 \pm 0.22	4.44 \pm 0.32*
Yellow	<i>Streptococcus intermedius</i>	0.52 \pm 0.13	3.96 \pm 0.22*
Yellow	<i>Streptococcus mitis</i>	1.35 \pm 0.33	8.32 \pm 0.52*
Yellow	<i>Streptococcus oralis</i>	1.51 \pm 0.42	10.89 \pm 0.53*
Yellow	<i>Streptococcus sanguis</i>	0.41 \pm 0.09	3.26 \pm 0.25*
Green	<i>Aggregatibacter actinomycetemcomitans</i>	0.08 \pm 0.02	0.17 \pm 0.02*
Green	<i>Capnocytophaga gingivalis</i>	0.08 \pm 0.02	0.04 \pm 0.01
Green	<i>Capnocytophaga ochracea</i>	0.24 \pm 0.04	0.70 \pm 0.03*
Green	<i>Capnocytophaga sputigena</i>	0.25 \pm 0.03	0.39 \pm 0.02*
Green	<i>Eikenella corrodens</i>	0.34 \pm 0.06	1.07 \pm 0.04*
Orange	<i>Campylobacter gracilis</i>	0.12 \pm 0.03	0.18 \pm 0.01
Orange	<i>Campylobacter rectus</i>	0.08 \pm 0.02	0.17 \pm 0.02*
Orange	<i>Campylobacter showae</i>	0.32 \pm 0.06	1.10 \pm 0.06*
Orange	<i>Eubacterium nodatum</i>	0.14 \pm 0.02	0.38 \pm 0.03*
Orange	<i>Fusobacterium nucleatum ss nucleatum</i>	0.41 \pm 0.08	1.24 \pm 0.06*
Orange	<i>Fusobacterium nucleatum ss polymorphum</i>	0.30 \pm 0.04	0.87 \pm 0.06*
Orange	<i>Fusobacterium nucleatum ss vincentii</i>	0.39 \pm 0.06	1.53 \pm 0.08*
Orange	<i>Fusobacterium periodonticum</i>	0.20 \pm 0.04	0.89 \pm 0.11*
Orange	<i>Parvimonas micra</i>	0.26 \pm 0.04	0.92 \pm 0.05*
Orange	<i>Prevotella intermedia</i>	0.08 \pm 0.02	0.07 \pm 0.01
Orange	<i>Prevotella nigrescens</i>	0.12 \pm 0.03	0.19 \pm 0.01
Orange	<i>Streptococcus constellatus</i>	0.34 \pm 0.07	2.85 \pm 0.20*
Red	<i>Tannerella forsythia</i>	0.07 \pm 0.02	0.03 \pm 0.00
Red	<i>Porphyromonas gingivalis</i>	0.26 \pm 0.05	1.13 \pm 0.05*
Red	<i>Treponema denticola</i>	0.23 \pm 0.06	0.49 \pm 0.06*
Others	<i>Eubacterium saburreum</i>	0.46 \pm 0.08	2.35 \pm 0.15*
Others	<i>Gemella morbillorum</i>	0.69 \pm 0.10	1.91 \pm 0.06*
Others	<i>Leptotrichia buccalis</i>	0.43 \pm 0.04	0.93 \pm 0.04*
Others	<i>Neisseria mucosa</i>	0.51 \pm 0.08	1.42 \pm 0.07*
Others	<i>Propionibacterium acnes</i>	0.70 \pm 0.07	0.40 \pm 0.10
Others	<i>Prevotella melaninogenica</i>	0.10 \pm 0.03	0.07 \pm 0.01
Others	<i>Streptococcus anginosus</i>	0.25 \pm 0.06	1.72 \pm 0.12*
Others	<i>Selenomonas noxia</i>	0.42 \pm 0.08	1.36 \pm 0.05*
Others	<i>Treponema socranskii</i>	0.12 \pm 0.03	0.38 \pm 0.03*

*The cell counts between day0 and day20 were significantly different ($p < 0.05$, paired Student's t-test, $n=6$)

Table 2.3 Mean counts (x 10⁵) (Mean±SEM) of 40 species in the treatment phase

Group/Visit		Vehicle/Day20 (x 10 ⁵)	RvE1/Day20 (x 10 ⁵)	Vehicle/Day40 (x 10 ⁵)	RvE1/ Day 40 (x 10 ⁵)
Complex	Species				
Actinomyces	<i>Actinomyces gerencseriae</i>	2.22±0.26	2.00±0.07	6.45±0.70	4.91±0.36
Actinomyces	<i>Actinomyces israelii</i>	0.67±0.12	0.51±0.04	2.43±0.25	1.78±0.15
Actinomyces	<i>Actinomyces naeslundii I</i>	2.68±0.38	2.30±0.09	3.16±0.38	2.22±0.18
Actinomyces	<i>Actinomyces viscosus</i>	1.06±0.12	0.90±0.05	2.89±0.29	2.23±0.16
Purple	<i>Actinomyces odontolyticus</i>	1.54±0.15	1.33±0.04	4.94±0.44	3.88±0.35
Purple	<i>Veillonella parvula</i>	11.40±0.19	12.10±0.17	18.63±0.98	17.93±0.52
Yellow	<i>Streptococcus gordonii</i>	4.22±0.36	5.49±0.35	12.66±0.47	11.50±0.96
Yellow	<i>Streptococcus intermedius</i>	3.57±0.30	4.56±0.24	9.39±0.28	6.74±0.66
Yellow	<i>Streptococcus mitis</i>	7.92±0.67	9.66±0.76	15.92±0.89	17.81±0.51
Yellow	<i>Streptococcus oralis</i>	10.50±0.73	12.09±0.67	20.04±1.50	21.56±0.72
Yellow	<i>Streptococcus sanguis</i>	3.48±0.26	3.86±0.31	8.02±0.41	6.65±0.62
Green	<i>Aggregatibacter actinomycetemcomitans</i>	0.23±0.03	0.19±0.02	1.36±0.03	0.95±0.07*
Green	<i>Capnocytophaga gingivalis</i>	0.05±0.01	0.05±0.01	1.10±0.03	0.76±0.01*
Green	<i>Capnocytophaga ochracea</i>	0.69±0.07	0.74±0.01	2.27±0.13	1.96±0.12
Green	<i>Capnocytophaga sputigena</i>	0.39±0.00	0.42±0.02	1.73±0.03	1.36±0.03*
Green	<i>Eikenella corrodens</i>	1.05±0.06	1.17±0.03	3.02±0.15	2.12±0.11*
Orange	<i>Campylobacter gracilis</i>	0.19±0.02	0.22±0.02	1.17±0.04	0.82±0.03*
Orange	<i>Campylobacter rectus</i>	0.19±0.02	0.21±0.01	1.17±0.05	0.76±0.03*
Orange	<i>Campylobacter showae</i>	1.28±0.00	1.12±0.03	3.36±0.15	2.29±0.06*
Orange	<i>Eubacterium nodatum</i>	0.39±0.03	0.46±0.02	1.56±0.04	1.17±0.01*
Orange	<i>Fusobacterium nucleatum ss nucleatum</i>	1.30±0.06	1.35±0.09	4.83±0.63	3.15±0.29
Orange	<i>Fusobacterium nucleatum ss polymorphum</i>	0.94±0.07	0.90±0.08	4.46±0.80	3.03±0.39
Orange	<i>Fusobacterium nucleatum ss vincentii</i>	1.58±0.07	1.73±0.12	4.73±0.58	2.84±0.15
Orange	<i>Fusobacterium periodonticum</i>	1.12±0.03	0.95±0.17	6.16±1.71	4.21±0.97
Orange	<i>Parvimonas micra</i>	0.98±0.04	1.03±0.04	2.64±0.11	1.83±0.05*
Orange	<i>Prevotella intermedia</i>	0.07±0.01	0.08±0.01	0.92±0.06	0.46±0.02*
Orange	<i>Prevotella nigrescens</i>	0.18±0.02	0.20±0.01	1.43±0.04	1.07±0.02*
Orange	<i>Streptococcus constellatus</i>	2.85±0.16	3.42±0.27	7.71±0.33	5.29±0.38*
Red	<i>Tannerella forsythia</i>	0.03±0.01	0.03±0.00	0.85±0.03	0.48±0.02*
Red	<i>Porphyromonas gingivalis</i>	1.15±0.08	1.25±0.06	3.42±0.16	2.02±0.06*
Red	<i>Treponema denticola</i>	0.35±0.05	0.64±0.08	4.22±1.05	2.15±0.30
Others	<i>Eubacterium saburreum</i>	2.48±0.16	2.71±0.16	6.49±0.29	3.48±0.04*
Others	<i>Gemella morbillorum</i>	1.89±0.06	2.08±0.08	5.65±0.17	4.26±0.03*
Others	<i>Leptotrichia buccalis</i>	1.05±0.03	0.94±0.03	2.43±0.09	2.03±0.06
Others	<i>Neisseria mucosa</i>	1.63±0.06	1.46±0.05	5.09±0.23	3.54±0.15*
Others	<i>Propionibacterium acnes</i>	0.55±0.21	0.30±0.09	1.48±0.08	1.20±0.07
Others	<i>Prevotella melaninogenica</i>	0.05±0.01	0.10±0.02	1.42±0.13	1.37±0.04
Others	<i>Streptococcus anginosus</i>	1.80±0.10	2.02±0.13	4.55±0.28	3.20±0.17*
Others	<i>Selenomonas noxia</i>	1.28±0.08	1.53±0.03	4.17±0.34	2.90±0.10
Others	<i>Treponema socranskii</i>	0.41±0.02	0.41±0.02	1.67±0.09	1.11±0.02*

*Mean cell counts between the RvE1 group (n=3) and the Vehicle group (n=3) on d40 was significantly different (p<0.05, Student's t test)

Table 2.4 Relative proportions of periodontal microbial complexes in the disease induction phase

Visit	Day 0	Day 8	Day 12	Day 16	Day 20
^aComplex	(%)	(%)	(%)	(%)	(%)
Actinomyces	15.2	6.5	8.0	8.0	8.9
Purple	8.4	13.5	16.8	16.2	17.4
Yellow	27.6	41.4	43.3	42.4	40.8
Green	6.4	4.5	3.3	3.6	3.1
Orange	16.8	15.4	11.7	13.4	13.7
Red	3.4	2.6	1.6	2.0	2.2
Others	22.3	15.9	15.3	14.5	13.9
Total	100	100	100	100	100

^aThe definition of periodontal microbial complex was described in the previous publications (Socransky et al., 1998, Socransky and Haffajee, 2005)

Table 2.5 Relative proportions of periodontal microbial complexes between the Vehicle group and the RvE1 group

Group/Visit	Vehicle/Day20	RvE1/Day20	Vehicle/Day 40	RvE1/ Day 40
Complex	(%)	(%)	(%)	(%)
Actinomyces	8.79	6.93	7.64	7.01
Purple	17.16	16.28	12.05	13.71
Yellow	39.36	43.21	33.76	40.41
Green	3.20	3.11	4.85	4.50
Orange	14.71	14.15	20.53	16.92
Red	2.03	2.33	4.34	2.93
Others	14.76	14.00	16.85	14.52
Total	100	100	100	100

Table 2.6 Relative abundance of phylum level during the treatment phase

Group/Visit	Vehicle/D20	RvE1/D20	Vehicle/D32	RvE1/D32	Vehicle/D40	RvE1/D40
Phylum	(%)	(%)	(%)	(%)	(%)	(%)
Actinobacteria	14.8±6.6	10.0±2.0	10.8±1.1	15.5±5.0	52.3±2.5	19.9±10.3
Firmicutes	84.0±7.0	89.5±1.8	87.9±2.2	84.1±5.1	46.1±1.4	79.1±18.4
Fusobacteria	0.1±0.1	0.2±0.2	0.2±0.2	0.1±0	0.7±0.7	0.4±0.2
Proteobacteria	0.6±0.4	0.3±0.1	1.0±1.0	0.2±0.2	1.0±0.4	0.7±0.3
Verrucomicrobia	0.4±0.4	0±0	0±0	0±0	0±0	0±0

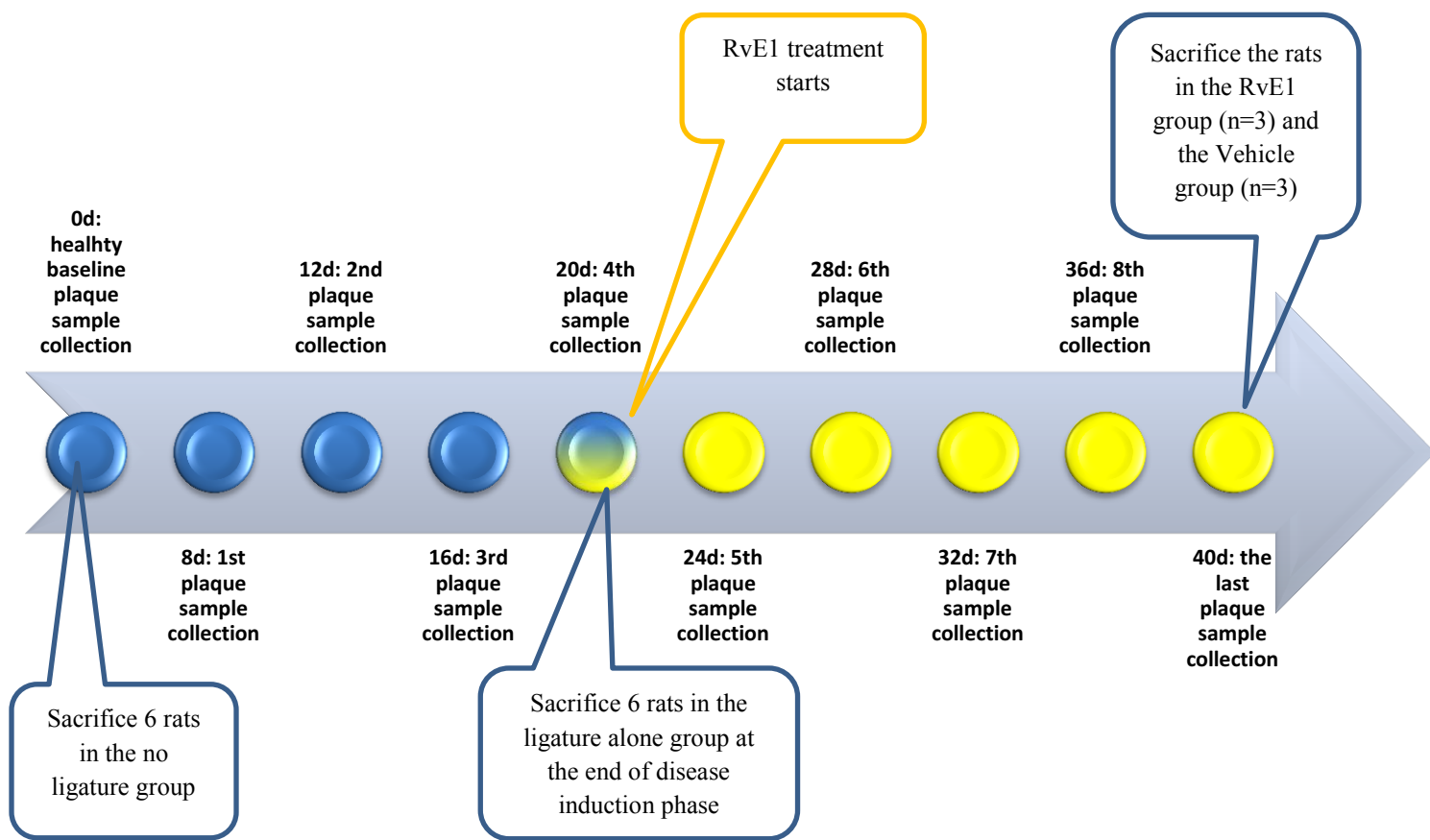
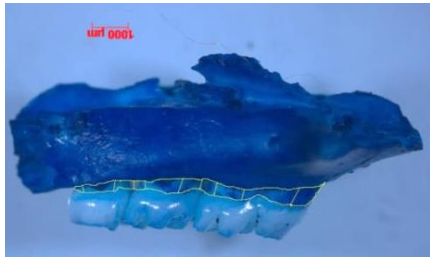


Figure 2.1: Experimental Timeline. Eighteen rats were assigned to four groups (no ligature: n=6, ligature alone: n=6, ligature + Vehicle: n=3, ligature + RvE1: n=3). The first three weeks of the experiment were the disease induction phase and the subsequent three weeks of the experiment were the treatment phase. RvE1 (0.25 μ g/ μ l) or vehicle (ethanol only) was applied to the ligated teeth every other day during the treatment phase. Subgingival plaque samples were collected from one tooth of each rat at each time point (day 0, 8, 12, 16, 20, 24, 28, 32, 36, 40).



Buccal



Palatal

Figure 2.2: Bone morphometric analysis: exposed root surface & distance between CEJ and alveolar bone. The exposed root surfaces on the buccal and palatal of three maxillary molars were measured. The distance between alveolar bone margin and cemento-enamel junction (CEJ) was measured at the nine indicated sites.

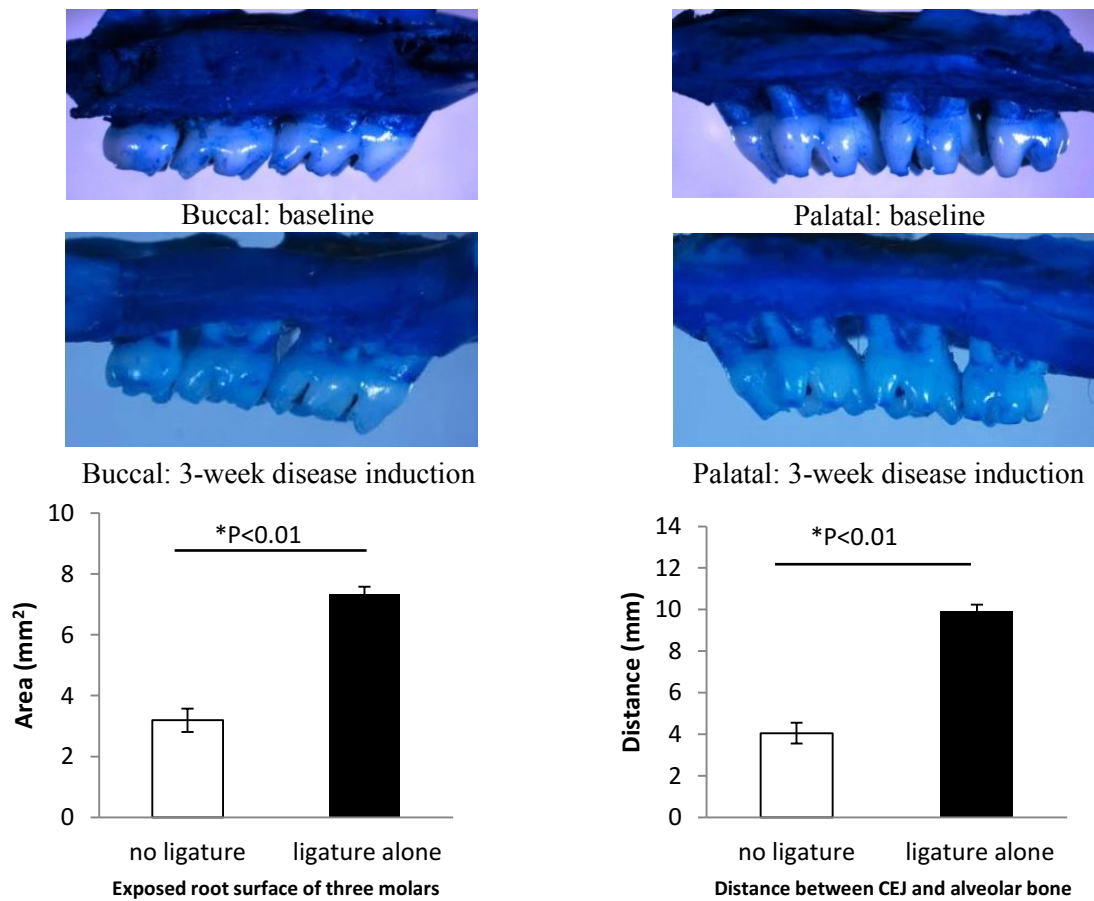


Figure 2.3: Alveolar bone level at baseline and three weeks after disease induction. Alveolar bone destruction was seen following three weeks of ligature placement in rats. The exposed root surface and the distance between CEJ and alveolar bone in the ligature alone group were significantly larger than these in the no ligature group.

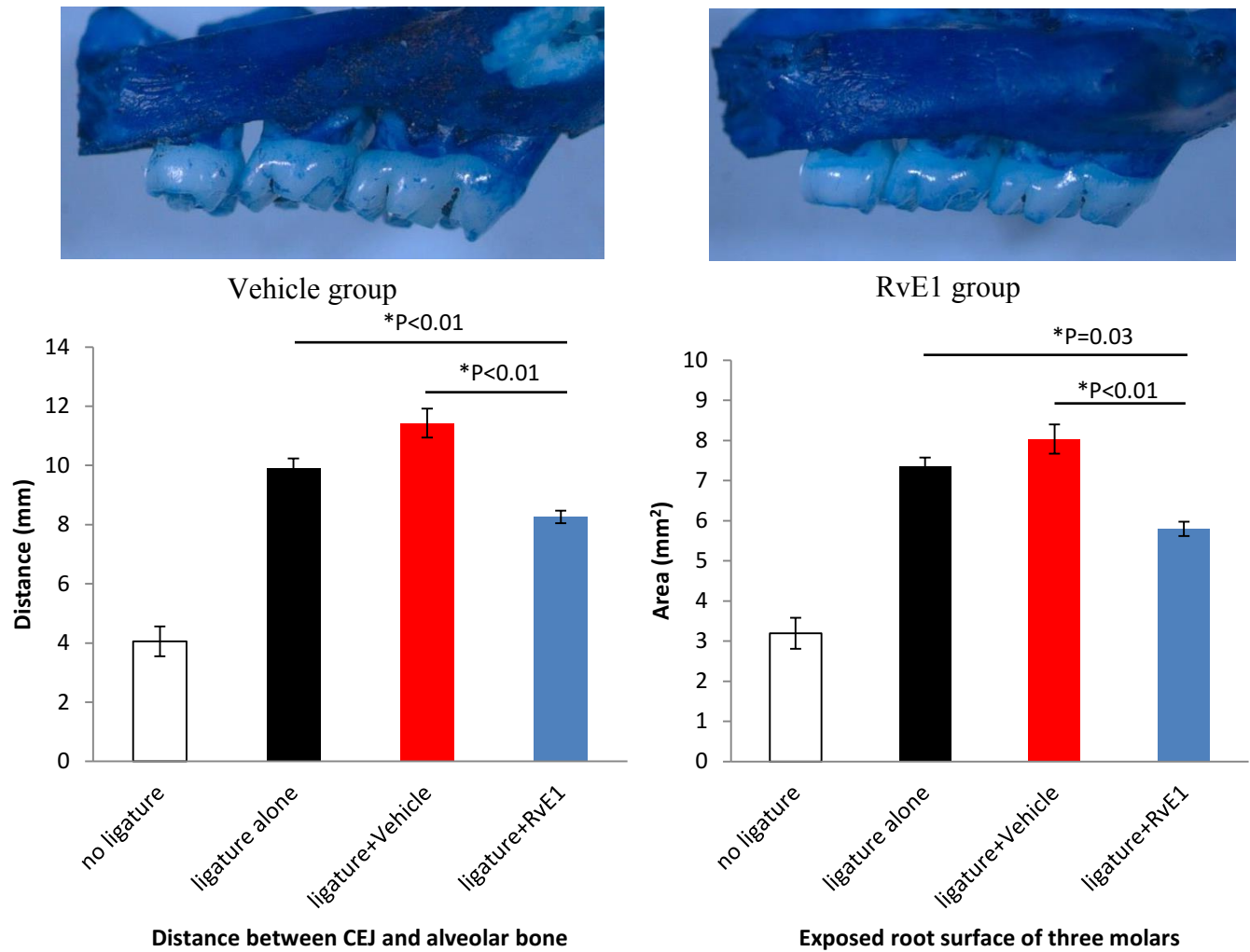


Figure 2.4: Bone morphometric analysis. The exposed root surface of the molars in the RvE1 group was significantly smaller than that in the Vehicle and the ligature alone group. The distance between CEJ and alveolar bone in the RvE1 group was also significantly shorter than that in the Vehicle group and the ligature group. RvE1 treatment significantly reversed alveolar bone loss associated with experimental periodontitis. The p-value was calculated by pair-wise t-test following one-way analysis of variance (ANOVA).

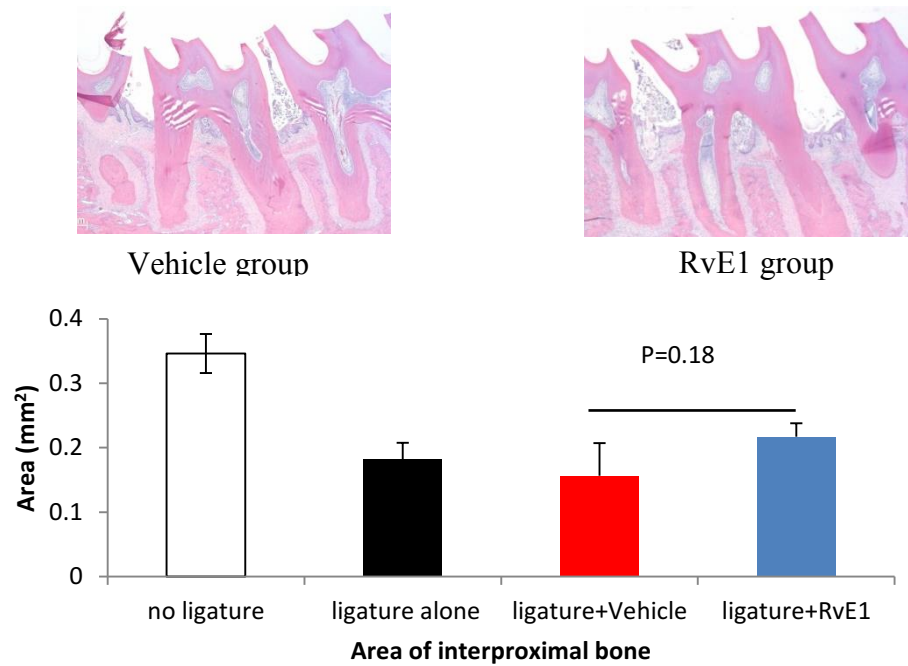


Figure 2.5: Histological area of interproximal bone. The area of interproximal bone in the RvE1 group was larger than that in the ligature alone group and the Vehicle group ($p=0.18$). The p -value was calculated by pair-wise t -test following one-way analysis of variance (ANOVA).

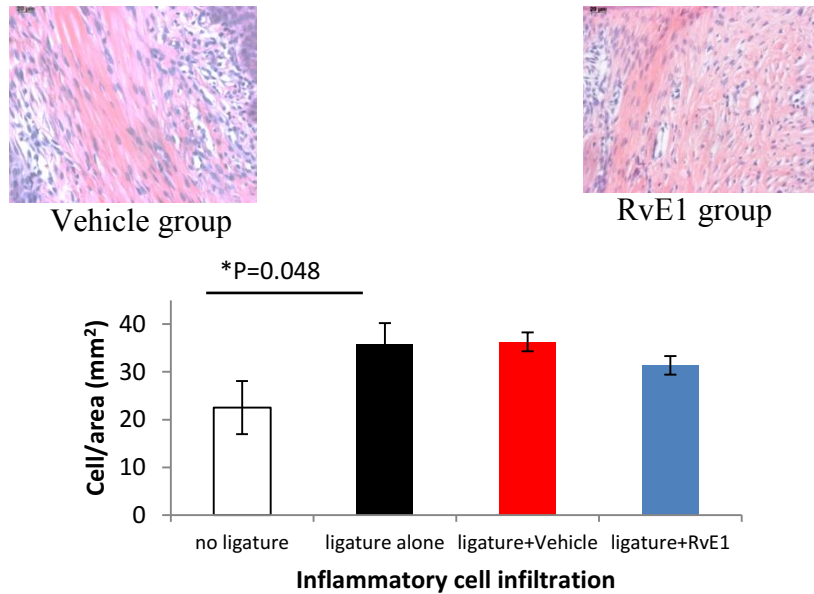


Figure 2.6: Inflammatory cell infiltrate. Inflammatory cell counts in the RvE1 group tended to be lower than that in the ligature alone group and the Vehicle group. However, the differences were not statistically significant. There was a significantly greater inflammatory cell infiltrate in the ligature alone group compared to no ligature. The p-value was calculated by pair-wise t-test following one-way analysis of variance (ANOVA).

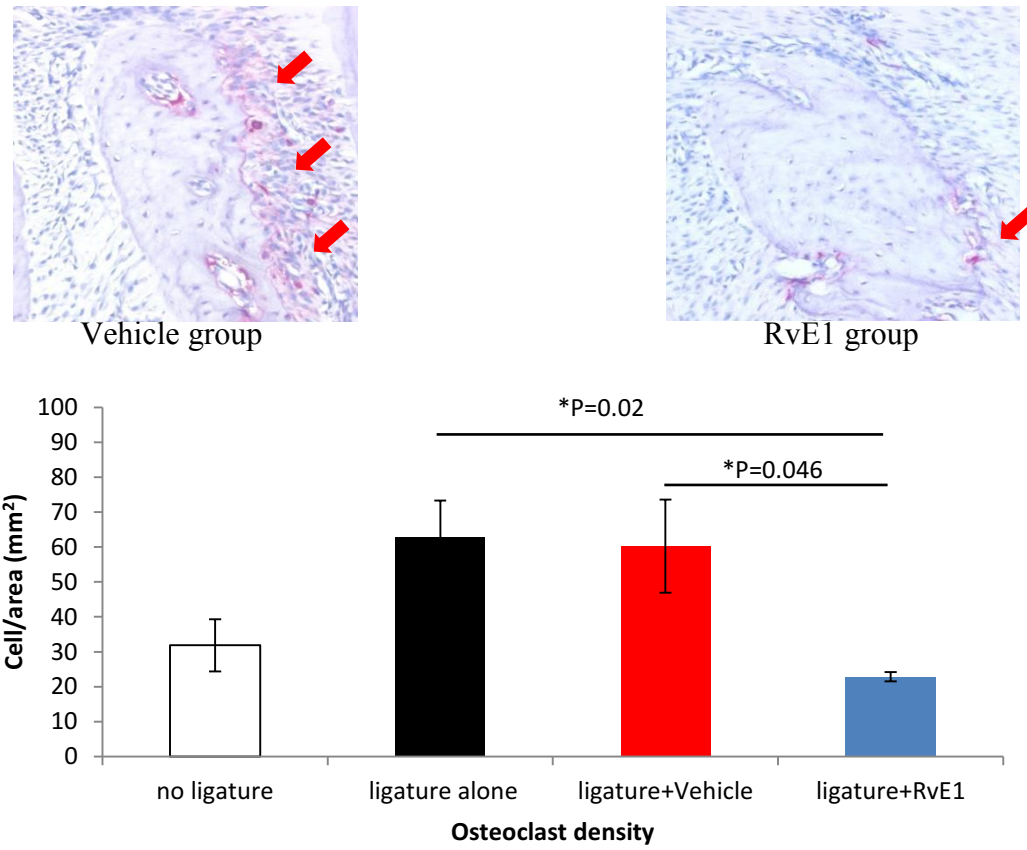


Figure 2.7: Osteoclast density. Osteoclast density was defined by dividing active osteoclast count (TRAP positive multinucleated osteoclasts) around the interproximal bone by the total area of the interproximal bone. The osteoclast density in the RvE1 group was significantly lower than that in the Vehicle and the ligature alone group. The p-value was calculated by pair-wise t-test following one-way analysis of variance (ANOVA).

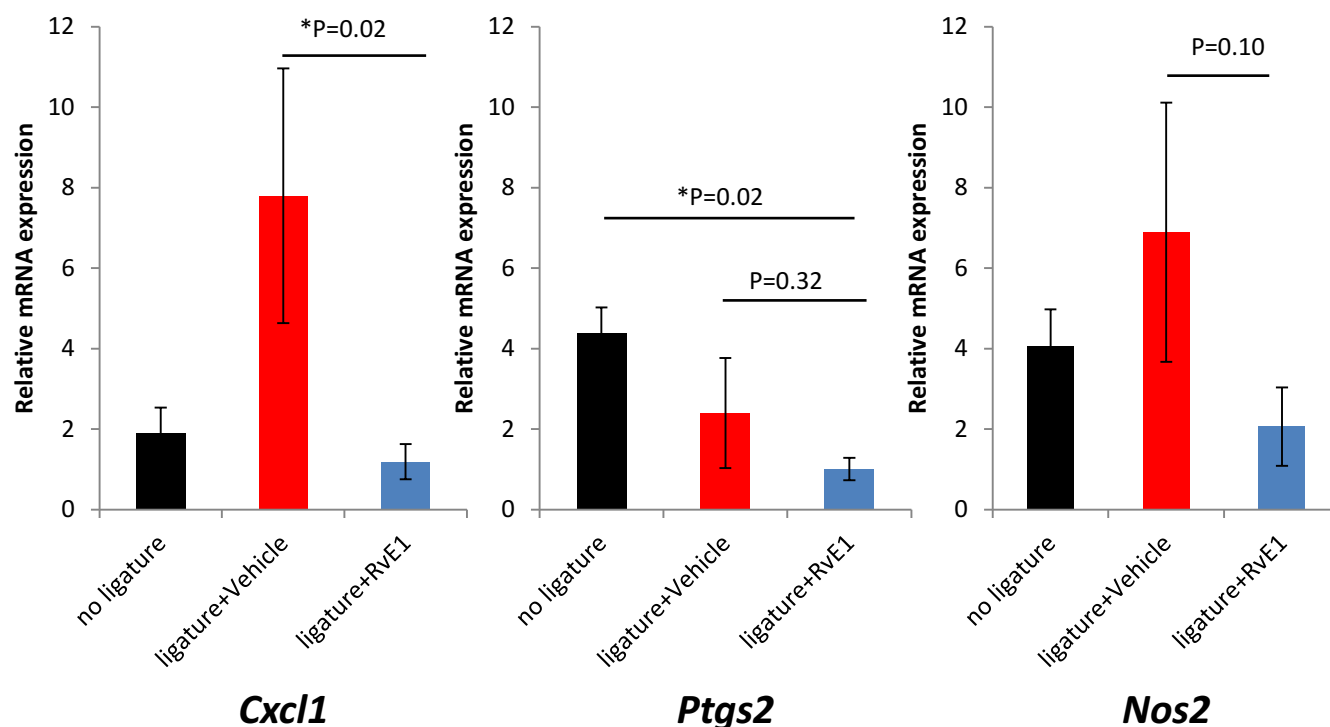


Figure 2.8: Relative quantity of inflammation-related gene expression ($2^{-\Delta\Delta CT}$). Relative mRNA expression ($2^{-\Delta\Delta CT}$) was used to analyze the gene expression levels of inflammation related genes in different groups. Gene expression of *Cxcl1* in the RvE1 group was significantly lower than that in the Vehicle group. Gene expression of *Ptgs2* in the RvE1 group was significantly lower than that in the ligature alone group. Gene expression of *Nos2* in the RvE1 group was lower than that in the ligature alone group and the Vehicle group but was not statistically significant. The p-value was calculated by pair-wise t-test following one-way analysis of variance (ANOVA).

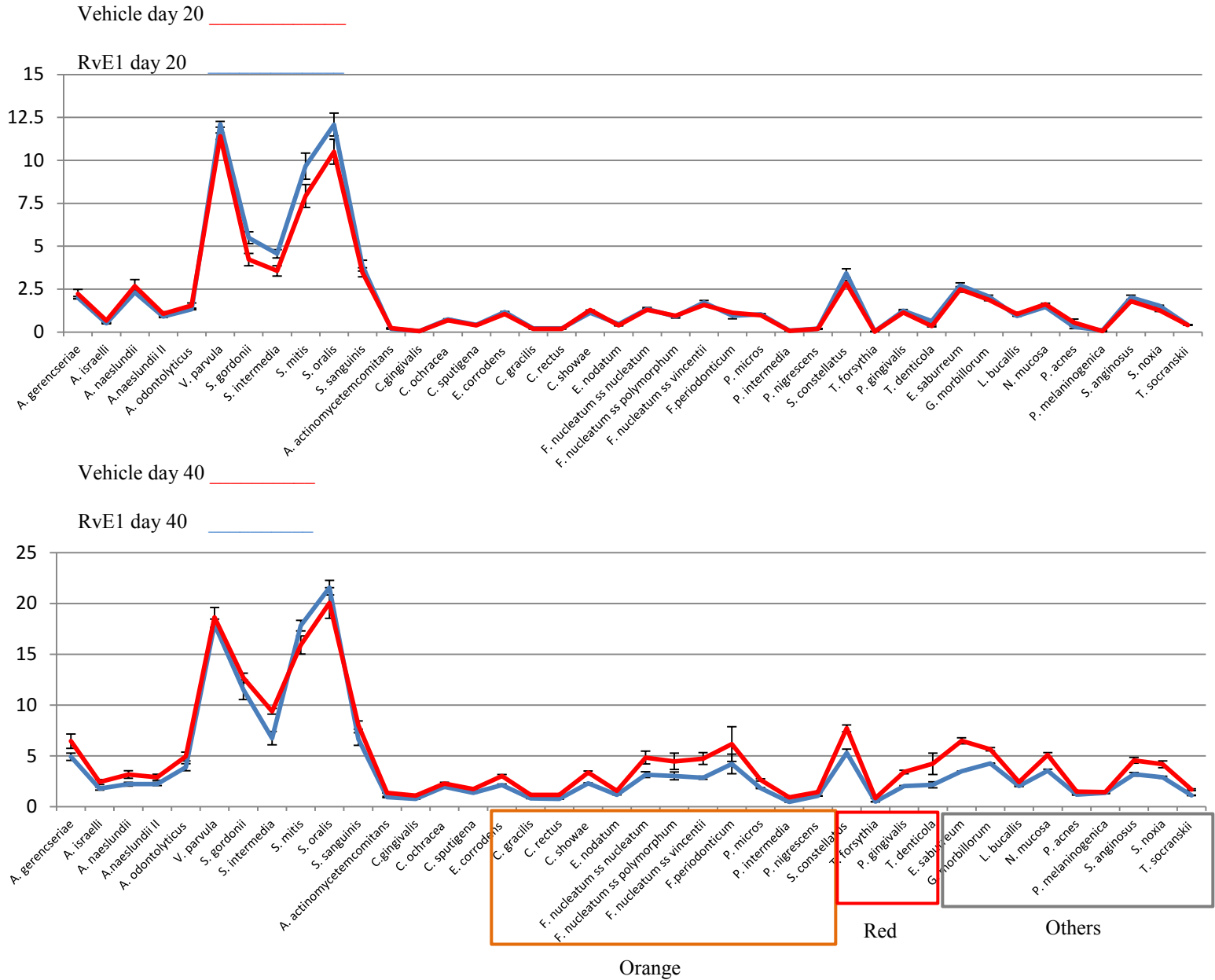


Figure 2.9: Mean counts ($\times 10^5$) of subgingival taxa on day 20 and day 40. At the baseline of the treatment phase (day 20), mean cell counts of most of the species had no significant difference between the Vehicle group and the RvE1 group. At the end of treatment phase (day 40), mean cell counts of species belonging to orange, red, or others complex were significantly reduced in the RvE1 group.

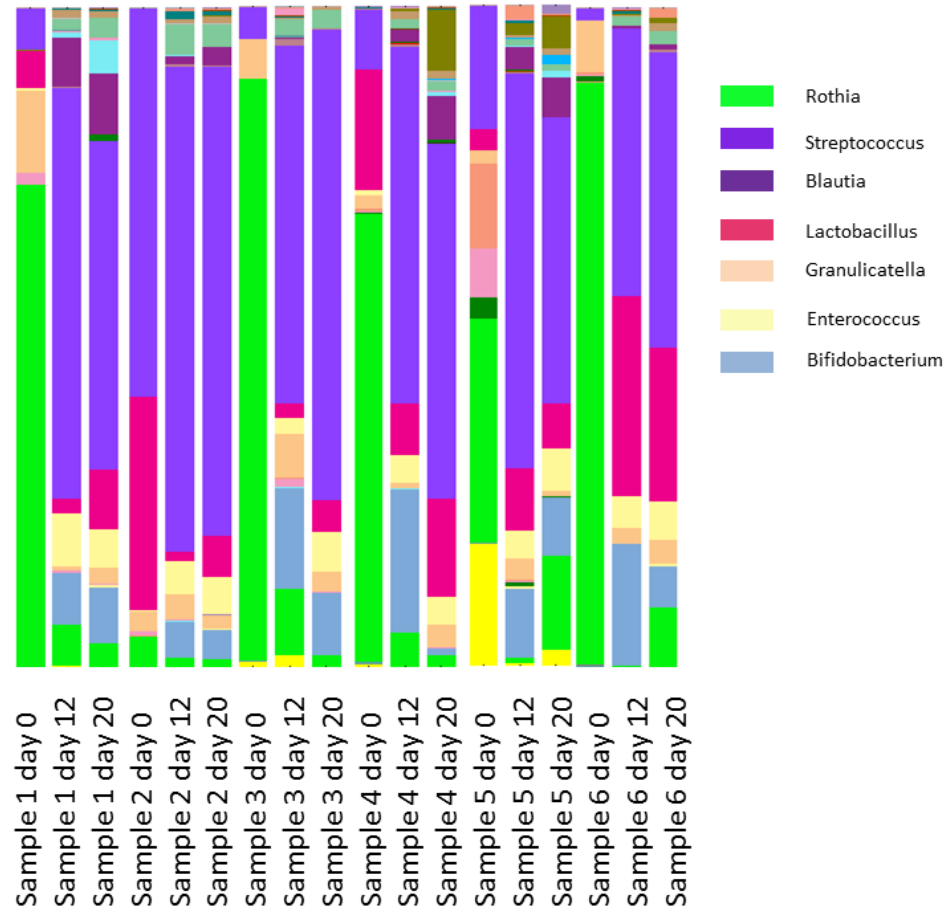


Figure 2.10: Taxonomic composition of the subgingival microbiota during disease induction phase (genus level). The taxonomic composition of subgingival microbiota in six samples is shown. The listed color-coded genera were the dominant genera during disease induction phase (day 0 to day 20). More information relating to color-coding taxonomy can be found in Appendix 1.

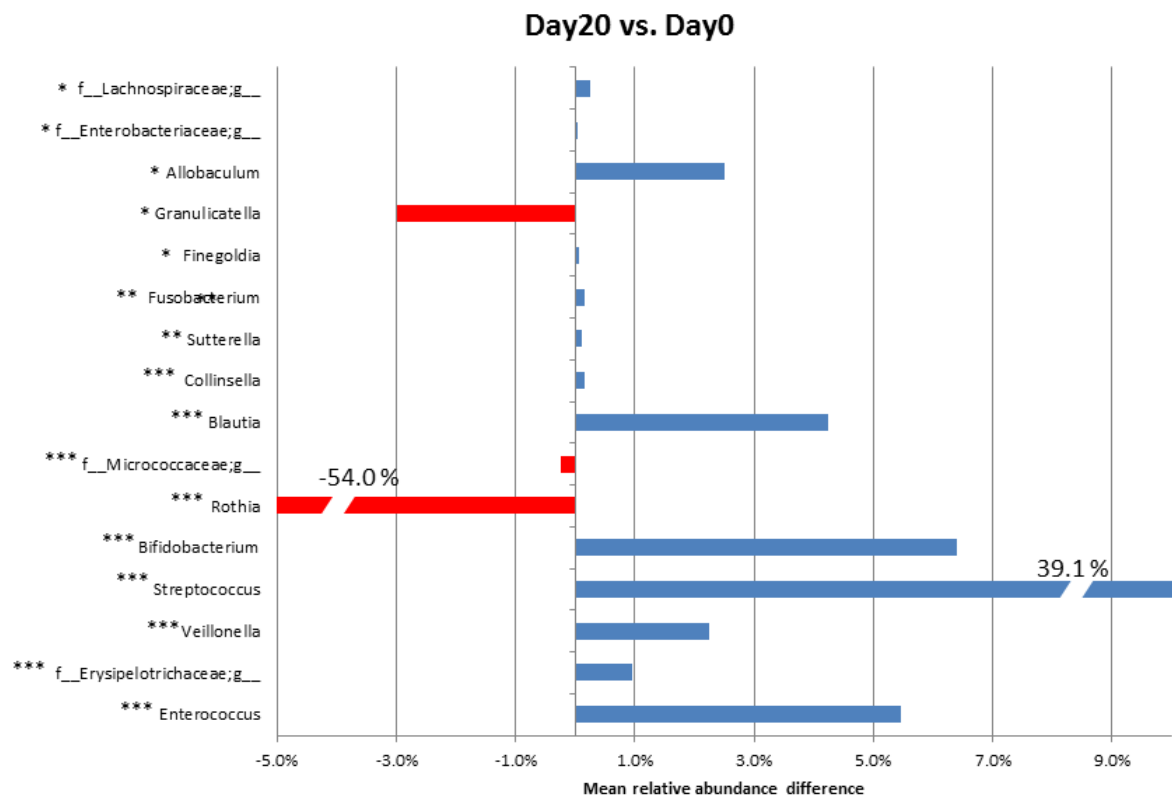
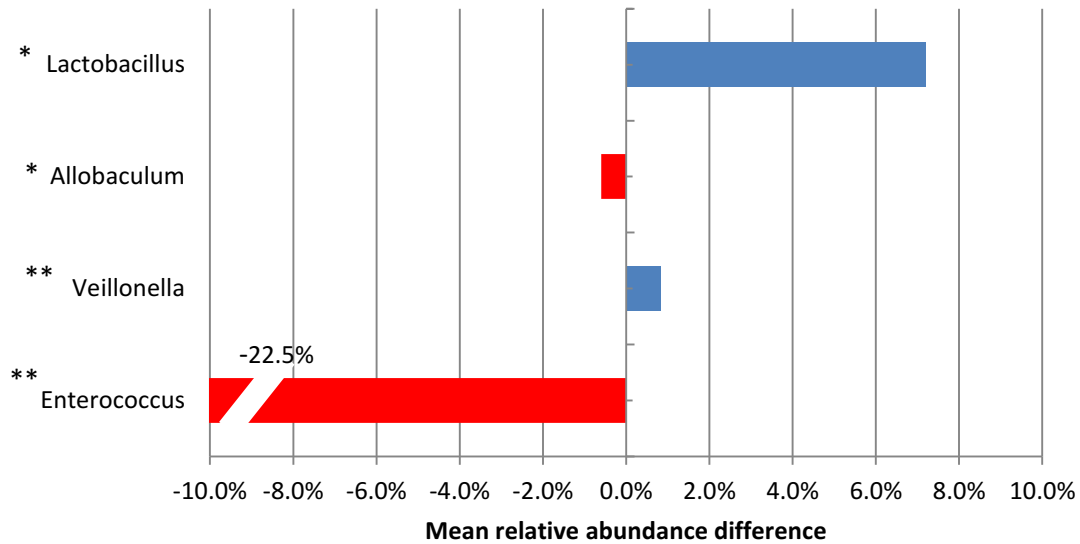


Figure 2.11: Mean difference of taxonomic relative abundance between day 0 and day 20. Mean relative abundance difference between day 0 and day 20 was calculated by subtracting mean relative abundance on day 0 from mean relative abundance on day 20 in the same sample. Positive values indicate the mean relative abundance on day 20 was larger than the relative abundance on day 0. Some OTUs did not match any known genera in the reference data base, therefore, only the names of family level (f__) were listed for these OTUs. The p-values were calculated by paired Student's t test (*** $P < 0.05$; ** $0.05 \leq P < 0.1$; * $0.1 \leq P < 0.2$).

RvE1 vs. Vehicle D32



RvE1 vs. Vehicle D40

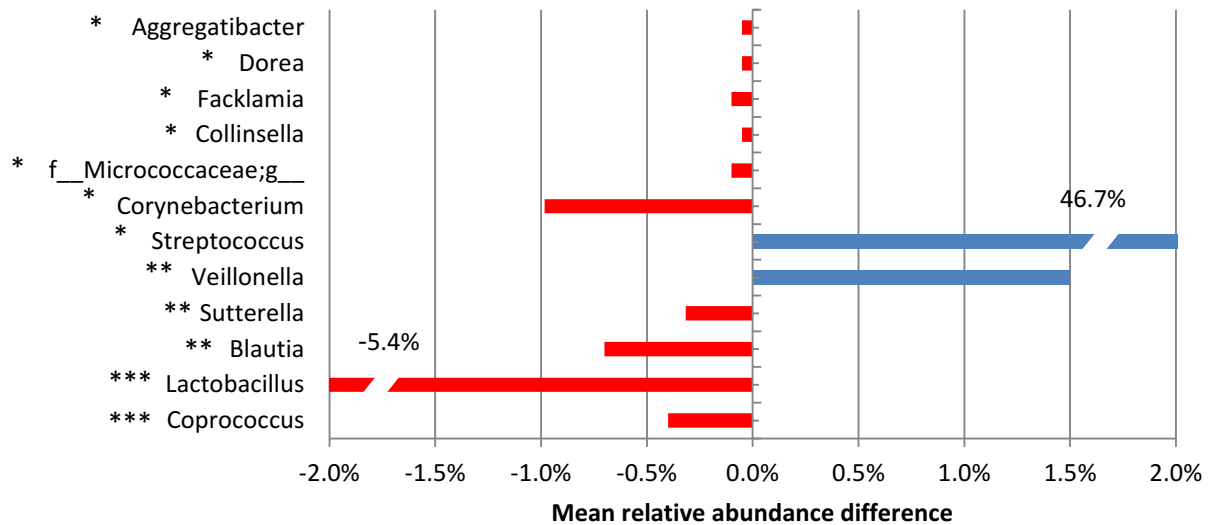


Figure 2.12: Mean difference of taxonomic relative abundance between the RvE1 group and the Vehicle group. Mean relative abundance difference between the RvE1 group and the Vehicle group was calculated by subtracting mean relative abundance of the Vehicle group from mean relative abundance of the RvE1 group. Positive values indicate the mean relative abundance of the RvE1 group was larger than the relative abundance of the Vehicle group. The p-values were calculated by Student's t test (*** $p < 0.1$; ** $0.1 \leq p < 0.2$; * $0.2 \leq p < 0.3$)

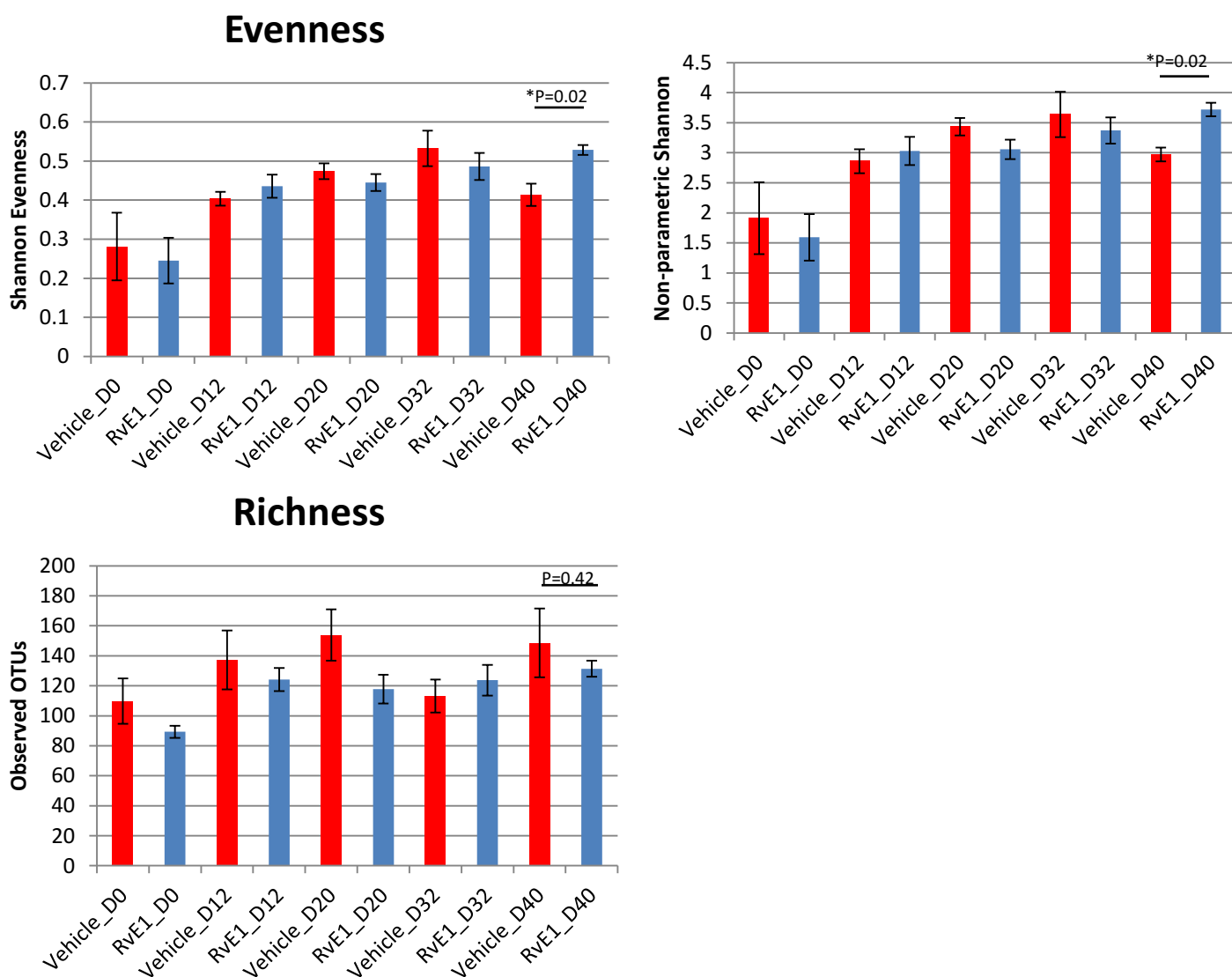


Figure 2.13: Alpha diversity of subgingival microbial community. The difference in Shannon evenness, non-parametric Shannon index and number of observed OTUs between the Vehicle group and the RvE1 group at each time point (n=3 in each group) was compared (Student's t-test). The Shannon evenness and non-parametric Shannon index in the Vehicle group were significantly lower than in the RvE1 group on day 40.

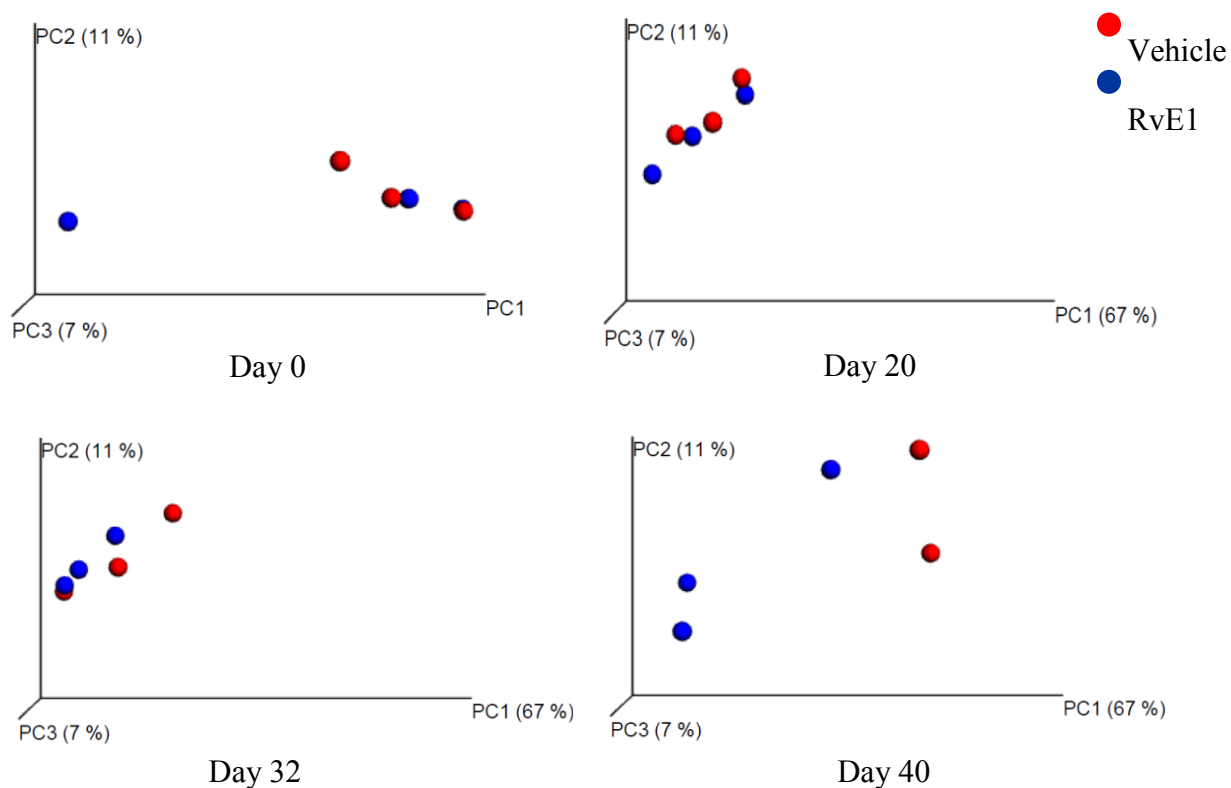


Figure 2.14: Principal coordinate analysis (PCoA). Principal coordinate analysis plots depicting distances among microbial communities based on qualitative community metrics show that the inflammation induced by ligature placement drove the composition change in the microbial community. The microbial community in the RvE1 group clustered apart from the microbial community in the Vehicle group at the end of treatment phase (day 40). Larger values of each component (PC) explain more of the variance in the data.

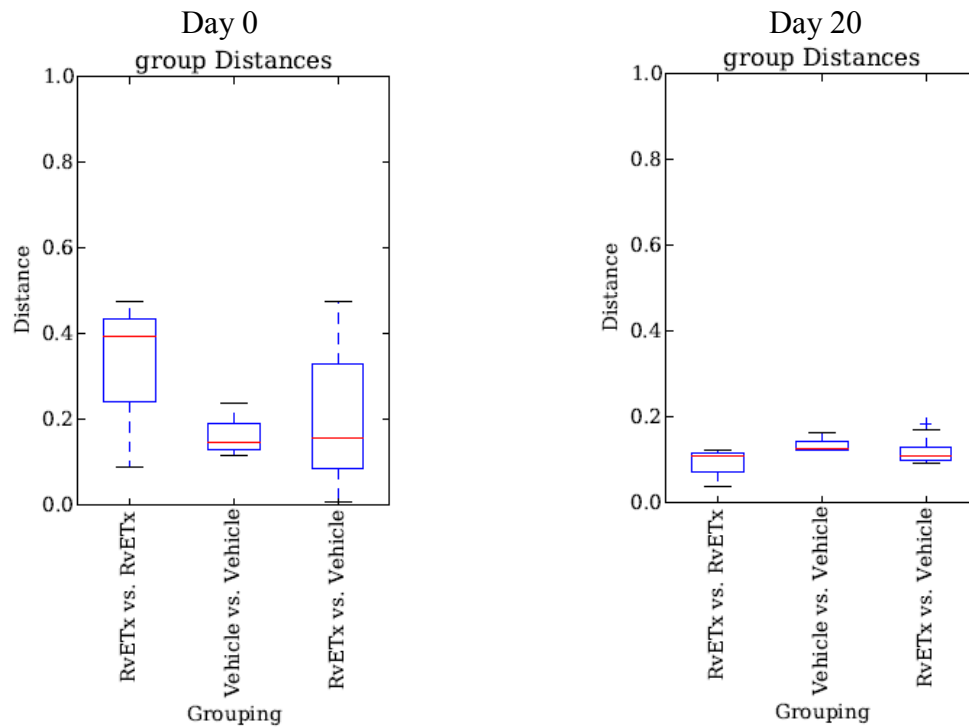


Figure 2.15: Beta diversity of subgingival microbial community during the disease induction phase. Distances were calculated by weighted UniFrac. RvETx vs. RvETx represents the distance between samples in the RvE1 group. Vehicle vs. Vehicle represents the distance between samples in the Vehicle group. RvETx vs. Vehicle represents the distances between samples in the RvE1 group and samples in the Vehicle group. There were no statistically significant differences between any group comparison ($p>0.05$).

Discussion

Regeneration of tissues lost to disease induced SPMs has been demonstrated in several models in addition to periodontitis (Serhan et al., 2012, Serhan, 2014), but the mechanism of regeneration induced by SPMs is not entirely clear. It is believed that enhanced recruitment of non-phlogistic macrophages in the resolution phase might play a critical role given that macrophages of the proper phenotype are necessary for various types of tissue regeneration (Yin et al., 2006, Godwin et al., 2013, Aurora et al., 2014). The present study suggests that RvE1 application regenerated alveolar bone lost to periodontitis that along with inhibition of inflammation and reduction of osteoclast activity. Regeneration of alveolar bone following SPM application has been demonstrated in a *P. gingivalis* induced periodontitis rabbit model (Hasturk et al., 2007) and a surgery and plaque induced periodontitis minipig model (Van Dyke et al., 2015). The present model had a relatively more horizontal bone loss induced by ligature placement compared to the bone loss in the rabbit or minipig model and thus presented a more challenging model to regenerate lost alveolar bone than the previous studies. It has been reported that the distance between CEJ and alveolar bone in the molar region of the rat gradually increases during physiological growth (Kuhr et al., 2004). Although this could not be controlled in this experiment, the potential for underestimation of the impact of RvE1 treatment exists. Taken together, the data suggest that RvE1 demonstrates promise as a topical therapy to regenerate tissues lost to periodontal inflammation.

It is well established that RvE1 inhibits neutrophil infiltration, osteoclast differentiation, and enhances efferocytosis of apoptotic cells during inflammation

resolution (Serhan et al., 2008). In the present study, RvE1 treatment significantly reduced osteoclast density compared to vehicle. The inhibition of immune cell infiltration and inflammation related gene expression were observed following RvE1 application. While all inflammation related genes tested were reduced, only the reduction in expression of *Cxcl1* was statistically significantly different from the Vehicle group at the end of RvE1 treatment. Given *Cxcl1* is a very important neutrophil chemoattractant in rodents, downregulation of the *Cxcl1* gene product likely plays a major role in reduction of local inflammation following RvE1 application. The peak of inflammation in the ligature model usually occurs in the first two weeks as reflected in the degree of alveolar bone destruction (de Lima et al., 2000). In the present study, therapy began at week 3 to ensure the periodontitis was chronic, mimicking the human situation.

The concept, a breakdown in the balance between “protective” bacterial species and “harmful” bacterial species, has been termed “dysbiosis”. Dysbiosis within the resident biofilm is associated with several inflammation related diseases in humans, such as inflammatory bowel disease, colitis, obesity, and cancer (Tamboli et al., 2004, Round and Mazmanian, 2009, Sobhani et al., 2011, Henao-Mejia et al., 2012). Periodontitis is one of the best characterized human diseases associated with dysbiosis, which is also known as a microbial-shift disease (Darveau, 2010, Jiao et al., 2014). Taxonomic composition and cell counts within the subgingival microbiota have been related to the status of periodontal disease in a myriad of studies (Socransky et al., 1998, Paster et al., 2001, Socransky and Haffajee, 2005, Teles et al., 2013). Periodontal treatment results in a shift within the microbiota back to a health-related subgingival microbiota. Stability of

the shift requires appropriate professional maintenance (Haffajee et al., 2006, Colombo et al., 2012). From an ecological point of view, the alpha diversity decreases, but the beta diversity increases in the resolved state of periodontal disease compared to the diversity in active periodontal disease (Griffen et al., 2012, Liu et al., 2012, Abusleme et al., 2013, Li et al., 2014, Shi et al., 2015). In active (untreated) periodontal disease, many disease-associated species are able to grow well by obtaining required nutrients from the inflamed environment. Therefore, the diseased microbial community is more diverse at the local site (alpha diversity) than is the healthy microbial community. However, the microbial community of different diseased samples becomes more similar (beta diversity) compared to the microbial community of different healthy samples because the diseased environment favors the growth of similar taxa.

In the present study, the changes in the subgingival microbiota were significant, following the induction of experimental periodontitis (Table 2.2, 2.4, Fig. 2.10, 2.11). The increased cell counts of species and shift of relative proportions of microbial complexes were significant based on the results of checkerboard DNA-DNA hybridization. Regarding the diversity of ecological community, the richness and evenness of microbial community increased during the disease induction phase. At the end of the treatment phase, the lower number of OTUs observed in the RvE1 group compared to the Vehicle group, coupled with our understanding of an increased alpha diversity with the onset of periodontal disease, suggests that RvE1 was associated with resolution of periodontal disease (Fig. 2.13). The microbial community became less

diverse at the end of disease induction compared to the microbial community of different samples at baseline (Fig. 2.15).

At the end of the treatment phase, RvE1 treatment significantly inhibited bacterial cell growth (Table 2.3), and reversed the shift of the relative proportions of periodontal microbial complexes (Table 2.5). The dominance of *Streptococcus* species in the subgingival microbiota following RvE1 treatment could be observed both in the results of checkerboard DNA-DNA hybridization and 16s rRNA gene sequencing (Table. 2.5, Fig. 12). The profile of the subgingival microbiota in the RvE1 group is different from the profile of the subgingival microbiota in the Vehicle group at the end of treatment phase (Table 2.6, Fig. 2.12, 2.14). Considering the difference of periodontal environment (Fig. 2.4, 2.7, 2.8) and the differences in the subgingival microbiota between the RvE1 group and the Vehicle group following RvE1 application, the change of subgingival microbiota appeared to be induced by changes in the local environment.

The purpose of the pilot studies using DNA Checkerboard analysis was to screen for microbial changes using a relatively less expensive and faster technique before the unbiased 16S DNA sequencing was used. The pilot studies served their purpose, but the information obtained beyond the observation that changes occurred should not be ignored. After experimental periodontitis was induced, the species or genera of the human red complex and orange complex were detected by checkerboard DNA-DNA hybridization, but they were barely detected by next-generation sequencing suggesting that the human probes were cross-reacting with rat associated species that are quite distinct. The ability of checkerboard DNA-DNA hybridization to detect bacteria in rat biofilm is not a new

observation since it was previously shown that 25 species were detected by human bacterial DNA probes in the biofilm collected from the rat ligature induced periodontitis model utilizing checkerboard DNA-DNA hybridization (Duarte et al., 2010). It is fair to say that the species of rat biofilm detected by checkerboard DNA-DNA hybridization are not the same species detected in human biofilm. However, the changes in counts of species and the change of relative proportions in clusters are likely valid.

Although identifying taxa by 16s rRNA gene sequencing can avoid the problem of cross reactions, interpretation of the relative abundance in the taxonomic composition is difficult. Further, OUT assignment based on existing reference databases such as SILVA and Greengenes using 97% similarity in sequences of the V3 and V4 regions of the 16S gene can also result in OUT misclassifications, even at the genus level. One should also take into account the fact that these databases were not build solely with sequences from murine microorganisms, increasing the chances of misclassifications. According to results published only in abstract form, a mock community consisting of the same amount of the 40 human periodontitis associated species could be detected accurately by checkerboard DNA-DNA hybridization, but not all the species were detected by 16s rRNA gene sequencing. Also, the relative abundance of these species was biased in the sequencing results (Teles F, 2015). The bias in sequencing might be caused by different copy numbers of 16s rRNA in different species, the primers used to amplified DNA, the relative composition of bacteria in a community, and the DNA extraction method (Diaz et al., 2012, Abusleme et al., 2014).

In order to describe the microbiota in rat oral biofilm comprehensively, it was necessary to use a next-generation sequencing technique. Because there is no rat oral bacterial DNA probe set available and there is lack of information on the rat subgingival microbiota, it was not possible to directly compare techniques. Since the threshold of 97% sequence similarity was used to assign the OTUs in the present study, only results of genus level were reported. At the end of the treatment phase, several genera had smaller mean relative abundance in the RvE1 group than the mean relative abundance in the Vehicle group (Fig. 2.12). Some of the genera, such as *Coprococcus*, *Blautia*, *Sutterella*, *Collinsella* and *Dorea*, were anaerobic and reduced relative abundance with RvE1 treatment. These genera might be the experimental periodontitis associated pathogens in rat ligature model; many of the periodontitis associated pathogens in humans are also anaerobic and have small relative abundance (Teles et al., 2013). The results indicate that the local environment became less favorable for multiple species to grow following the continuation of RvE1 application.

It is interesting to observe that *Streptococcus* was the dominant genus in the RvE1 group at the end of the treatment phase, but not in the Vehicle group. Since *Streptococcus* was the dominant genus caused by ligature placement, the results can be explained by the overgrowth of pathogens in the Vehicle group reducing the percentage of *Streptococcus*. The same phenomenon was also observed in the change of relative proportions of periodontal microbial complexes. Taken together these results indicate that the difference in periodontal environment created by control of inflammation resulted in the changes in taxonomic composition of the subgingival microbiota.

CHAPTER THREE: PREVENTION OF SUBGINGIVAL MICROBIOTA SHIFT FOLLOWING RESOLVIN E1 APPLICATION

Introduction

The “ecological plaque hypothesis” was proposed to describe and explain the dynamics between the host environment and the oral microbiota in healthy and diseased conditions from the ecological point of view (Marsh, 1994, Marsh, 2003). The hypothesis emphasizes there is a direct link between local environmental conditions and the activity and composition of the microbial community. The changes in local environment will trigger the changes in the microbiota, and vice versa. For example, as a periodontal pocket forms an environment is created favoring putative pathogen growth and expression of virulence factors. Flourishing periodontal pathogens compete with health-related pathogens leading to the shift in the microbiota. In order to prevent periodontitis, it is best to avoid the initial establishment of diseased environment (gingivitis) and thus prevent the deleterious microbial shift.

In Chapter 2, it was demonstrated that application of resolvin E1 (RvE1) reverses the shift in the subgingival microbiota by inhibiting inflammation and reversing bone loss in ongoing experimental periodontitis. The results imply the recovery of a diseased local environment leads the change of subgingival microbiota. If the ecological plaque hypothesis regarding the relationship between host environment and characteristics of the microbiota is true, prevention of formation of a diseased environment should stop the shift of the microbiota. In order to test this hypothesis, we proposed two specific aims:

Specific Aim 1 – To examine the changes of rat subgingival microbiota during periodontitis progression with or without prophylactic RvE1 treatment using next-generation sequencing

We hypothesize that prophylactic RvE1 application will prevent or reduce periodontitis progression in the ligature induced periodontitis rat model, and the subgingival microbiota composition will be relatively stable.

Specific Aim 2 – To examine the changes of the clinical and local inflammatory response in the periodontium of rats with or without prophylactic treatment

We hypothesize that the resolution of inflammation will prevent changes in the subgingival microbiota. Bone morphometry, histomorphometry, and real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) are used to analyze the change of local environment and inflammatory response.

Materials and methods

Animals and experimental periodontitis model

This experiment was approved by Institutional Animal Care and Use Committee (IACUC) of the Forsyth Institute. Twelve six-week old male Wistar rats (weight 180-200 g, Charles River Laboratories, New York, NY) were used, and all animals were housed in controlled temperature (22° C to 25° C) and dark/light cycle (12/12 hours) conditions. These rats received standard laboratory chow diet and water *ad libitum*. Rats were sedated by isoflurane 2-3% before being anesthetized with ketamine (80mg/kg, intraperitoneally) and xylazine (16 mg/kg, intraperitoneally).

Sixteen rats were divided into four groups (no ligature, ligature + Vehicle, ligature + RvE1 (0.1 μ g/ μ l), ligature + RvE1 (0.5 μ g/ μ l)), and each group had four rats. 5–0 silk ligature placed subgingivally on the first day of this experiment to induce periodontitis on the maxillary right and left second molars of all rats. The experimental period was four weeks. During the experiment, 1 μ g/ μ l RvE1 (Cayman Chemical Co. Inc., Ann Arbor, MI) dissolved in 9 μ l of saline was applied on the ligated teeth of the rats in the RvE1 (0.1 μ g/ μ l=0.28mM) group, 1 μ g/ μ l RvE1 dissolved in 1 μ l of saline was applied on the ligated teeth of the rats in the RvE1 (0.5 μ g/ μ l=1.4mM) group, and 10 μ l of 10% ethanol (vehicle alone) was applied on the ligated teeth of the rats in the Vehicle group. RvE1 solution and vehicle were applied three times a week (M, W, F). Four rats in the no ligature group were sacrificed on the first day of the experiment to collect healthy baseline tissue samples. At the end of four weeks, all rats in other groups were sacrificed to collect tissue samples. The timeline of the experiment is summarized in Fig. 3.1.

The collected samples included subgingival plaque, gingival tissue, maxillae, and serum. Subgingival plaque samples were collected on day 0, 7, 10, 14, 17, 21, 24, and 28. Other samples were collected immediately after sacrificing the rats. The maxillae were split into two halves: one half was taken for the analyses of bone morphometry and gingival gene expression. The palatal gingiva of the ligated molar was harvested and stored in 200 μ l of RNAlater (Sigma-Aldrich) for the qRT-PCR assay. The remaining specimen was defleshed by beetles to obtain clean bone block. The other half was processed for histomorphometric analysis. Blood was obtained by heart aspiration and

centrifuged to collect serum. Serum was aliquoted and stored at -80 °C (eight aliquots of 100µl each) for future analysis.

Bacterial DNA extraction and amplification

Subgingival plaque samples were collected by using the tip of 2-Whiteside scaler (Hu-Friedy, Chicago, IL) from the palatal site of the maxillary second molars. Before placing the ligature on the maxillary second molars, the plaque sample was collected from two teeth (two maxillary second molars) of each rat at baseline and then the samples were pooled in one collection tube. The sample was collected from one ligated tooth of each rat each time after ligature placement. The DNA of subgingival plaque was extracted using the MoYsis Basic (CaerusBio Inc., Downingtown, PA) and QIAamp® mini kit (QIAGEN Inc., Valencia, CA).

The extracted bacterial DNA was amplified with the multiple displacement amplification kit (GenomiPhi™ V3 DNA amplification kit, GE Healthcare Bio-Sciences, Pittsburgh, PA). The concentration of amplified DNA samples was measured with fluorescent nucleic acid stain (Quant-iT™ PicoGreen® dsDNA Assay, Life Technologies, Grand Island, NY).

Next-generation sequencing

The microbial composition of the plaque samples (50ng/10µl) were characterized by sequencing the hyper-variable V3 and V4 regions of the 16S rRNA gene using the Illumina MiSeq® platform. Successfully merged reads following paired-end sequencing were processed through the QIIME pipeline (Caporaso et al., 2010) filtering out low

quality sequences using a quality score threshold of 20. Operation Taxonomic Units (OTUs) were created by clustering the merged quality filtered reads at a 97% identity threshold using sequences from a reference database (SILVA/Greengenes) (DeSantis et al., 2006, Quast et al., 2013) as a guide. All remaining sequences entered a *de novo* clustering step where they were clustered at the same 97% identity threshold without guidance from the reference database. Potential chimeric OTUs were removed through the use of UCHIME (Edgar et al., 2011) and the gold 16 database (Public domain version of UCHIME, version 4.1, http://drive5.com/uchime/uchime_download.html). Taxonomic assignments to OTUs from phylum to genus level were completed through QIIME's use of the given reference database (SILVA/Greengenes) and the UCLUST algorithm (Edgar, 2010). Further analysis included alpha and beta diversity and abundance profiles of the microbial community in the samples.

Morphometric analysis

The dissected maxilla bones were stained with methylene blue and the images (in 0.63X10 times magnification) were taken under the dissecting microscope (Axio observer A1, ZEISS) using AxioVision 4.8 software. The areas of exposed root surface were measured at the buccal and palatal sites of three maxillary molars. The distance between alveolar bone margin and cemento-enamel junction (CEJ) was measured at nine sites (mesial, middle, and distal sites of the first molar; interproximal site between the first molar and the second molar; mesial and distal sites of the second molar; interproximal site between the second molar and the third molar; mesial and distal sites of the third molar) of three maxillary molars at the buccal site and the palatal site. The

direction of the distance line was parallel with the axis of root. All measurements were performed using computer software (ImageJ). The measurements of buccal and palatal sites were added for statistical analysis.

Histomorphometry

Half of the maxilla was processed following the protocol in chapter 2. Serial mesio-distal sections (6 μm) parallel to the long axis of the teeth were cut. Thin sections were either stained with H&E for light microscopy and identification of the cellular composition of inflammatory infiltrates, or stained with tartrate resistant acid phosphatase (TRAP) to examine osteoclastic activity.

Sections with similar anatomic positions were selected for quantitative measurements. Different sections were measurement randomly to avoid bias. The specific areas of mesial and distal interproximal sites were selected for histological assessment. Each measurement had three different sections. The number of inflammatory cells, including neutrophils, lymphocytes and plasma cells, was counted in the area of connective tissue above the alveolar bone at 400x magnification. The total number of osteoclasts associated with the interproximal bone was counted. The area of interproximal bone was measured. Osteoclast density was defined by dividing the number of osteoclasts by the area of interproximal bone. All the measurements were performed with computer software (ImageJ). The mean of the mesial site and the distal site from three sequential sections was determined.

Quantitative reverse transcription polymerase chain reaction assay

Total RNA was extracted from gingival tissue with Trizol reagent (Invitrogen, Grand Island, NY) as per the manufacturer's protocol. The concentration and purity of RNA was estimated by the A260/A280 ratio spectrophotometrically (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA). A total of 1 µg RNA was converted to cDNA using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Grand Island, NY).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using primers and TaqMan probes for *Cxcl1*, *Ptgs2*, *Nos2*, and labeled with FAM dye (Applied Bio-systems, TaqMan gene expression assays). β -actin (*Actb*) was utilized as an internal control and amplified using preformulated VIC-TAMRA-labeled TaqMan probes (Applied Biosystems, Endogenous Control). Quantification was performed in an automated thermal cycler (StepOnePlus™ System, Applied Biosystems). The reaction mixtures were kept at 50°C for two minutes (one cycle), 95°C for 20 seconds (one cycle), 95°C for one second and 60°C for 20 seconds (40 cycles). The results were analyzed through a software interface and spreadsheet for the calculation of relative expression ($2^{-\Delta\Delta CT}$).

Statistical Analysis

The comparison between two groups was analyzed by unpaired or paired two-tailed Student's t test. The comparison between multiple groups was analyzed by one-way analysis of variance (one-way ANOVA) and post hoc analysis was performed

(pairwise t-test). All values were expressed as mean \pm standard error of the mean. P-value <0.05 was considered statistically significant.

Results

Prophylactic RvE1 treatment prevents alveolar bone loss in the ligature induced periodontitis rat model

One rat in the RvE1 (0.5 μ g/ μ l) group died from the anesthesia in the second week of the experiment; the results of the RvE1 (0.5 μ g/ μ l) group were derived from three rats. Prophylactic RvE1 treatment significantly prevented alveolar bone loss compared to vehicle application (Mean area of exposed root surface in molars - ligature + Vehicle: 6.8 \pm 0.4 mm², ligature + RvE1 (0.1 μ g/ μ l): 5.3 \pm 0.3 mm², ligature + RvE1 (0.5 μ g/ μ l): 5.1 \pm 0.4 mm²; distance between CEJ and alveolar bone level- ligature + Vehicle: 8.9 \pm 0.5 mm, ligature + RvE1 (0.1 μ g/ μ l): 6.9 \pm 0.5 mm, ligature + RvE1 (0.5 μ g/ μ l): 6.5 \pm 0.4 mm) (Fig. 3.2). Mean histological area of interproximal bone in the RvE1 (0.1 μ g/ μ l) group and the RvE1 (0.5 μ g/ μ l) group was larger than the area in the Vehicle group (Fig.3.3).

Prophylactic RvE1 treatment inhibits inflammation and reduces osteoclast activity

Prophylactic RvE1 treatment significantly inhibited inflammatory cell infiltration compared to vehicle application. The cell counts in the connective tissue between the RvE1 (0.1 μ g/ μ l) group and the RvE1 (0.5 μ g/ μ l) group was not significantly different (Fig. 3.4). Osteoclast activity was significantly inhibited by prophylactic RvE1 treatment. Osteoclast density between the RvE1 (0.1 μ g/ μ l) group and the RvE1 (0.5 μ g/ μ l) group was not significantly different (Fig. 3.5).

Prophylactic RvE1 treatment (0.1 µg/µl) significantly inhibited the expression levels of *Ptgs2* and *Nos2* genes compared to vehicle application (Fig. 3.6). The gene expression level of *Ptgs2* in the RvE1 (0.5 µg/µl) group was significantly lower than that in the Vehicle group, but the gene expression level of *Nos2* in the RvE1 (0.5 µg/µl) group was lower than that in the Vehicle group with no statistical significance ($p=0.07$). Both RvE1 treatments (0.1 µg/µl and 0.5 µg/µl) did not significantly reduce the expression level of *Cxcl1* gene.

Subgingival microbiota change is inhibited by prophylactic RvE1 treatment

The taxonomic composition of subgingival microbiota at the genus level is demonstrated in Fig. 3.7. The taxonomic composition of the subgingival microbiota at baseline was significantly different from the compositions on day 14 and day 28. One genus, *Rothia*, exhibited dominant and consistent relative abundance in all the samples at baseline (72.6±6.6 %). After the disease was induced, the taxonomic composition became diverse in different samples. Generally, four genera, *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, had relatively higher relative abundance than other genera in samples during disease progression (Fig. 3.7).

While comparing the mean relative abundance of each genus in the RvE1 groups with the mean relative abundance of each genus in the vehicle group on day 14, several genera had a higher difference ($p<0.3$) between the RvE1 (0.5 µg/µl) group or the RvE1 (0.1 µg/µl) group and the Vehicle group than did other genera (Fig. 3.8.a&b). Among these genera, three genera, *Jeotgalicoccus*, *f__Enterococcaceae;g__*, *Haemophilus*, were

shared in the comparisons between the RvE1 groups (0.1 or 0.5 $\mu\text{g}/\mu\text{l}$) and the Vehicle group.

While comparing the mean relative abundance of each genus in the RvE1 groups with the mean relative abundance of each genus in the vehicle group on day 28, five genera (*f__Enterobacteriaceae;g__*, *f__Clostridiaceae;g__*, *f__Coriobacteriaceae;g__*, *Rothia*, *Granulicatella*) had a higher difference ($p<0.3$) between the RvE1 (0.1 $\mu\text{g}/\mu\text{l}$) group and the Vehicle group than did other genera, and eight genera (*f__Enterobacteriaceae;g__*, *Lactobacillus*, *Granulicatella*, *f__Micrococcaceae;g__*, *Turicibacter*, *f__Planococcaceae;g__*, *Facklamia*, *f__Clostridiaceae;Other*) had a higher difference ($p<0.3$) between the RvE1 (0.5 $\mu\text{g}/\mu\text{l}$) group and the Vehicle group than did other genera. Most of these genera had lower relative abundance in the RvE1 groups than the relative abundance in the Vehicle group (Fig. 3.9.a&b). Among these genera, *Granulicatella* and *f__Enterobacteriaceae* had lower mean relative abundance in both of the RvE1 groups than mean relative abundance in the Vehicle group.

The diversity of microbial community increased significantly after the disease was induced. Generally, the Vehicle group had higher evenness (Shannon evenness) and richness (number of observed OTUs) than did the RvE1 groups indicating more species grew in the inflamed environment without RvE1 application. Moreover, the Shannon evenness and the number of observed OTUs in each group on day 14 were higher than these on day 0 and day 28 (Fig. 3.10).

The subgingival microbiota in all three groups shifted significantly following the induction of periodontitis. The diversity between samples in three groups became larger

on day 14 and day 28 compare to the diversity between samples on day 0 (Fig. 3.11). At the end of experiment, the mean weighted UniFrac distance between samples in the Vehicle was shorter than the mean weighted UniFrac distances between samples in RvE1 groups without statistically significant differences (Fig. 3.12). The results indicate the phylogenetic profile of subgingival microbiota between samples in the Vehicle group was closer to each other than that in the RvE1 groups.

RvE1 application appeared to be correlated to the body weight change of animals

The mean increased body weight of rats in the RvE1 (0.5µg/µl) group was significantly higher than the mean increased body weight of rats in the RvE1 (0.1µg/µl) group and in the Vehicle group during the experimental period (four weeks). The mean increased body weight of rats in the Vehicle group was lower than the mean increased body weight of rats in the RvE1 groups (Fig.3.13). The results implied RvE1 application might be correlated to the body weight change of animals.

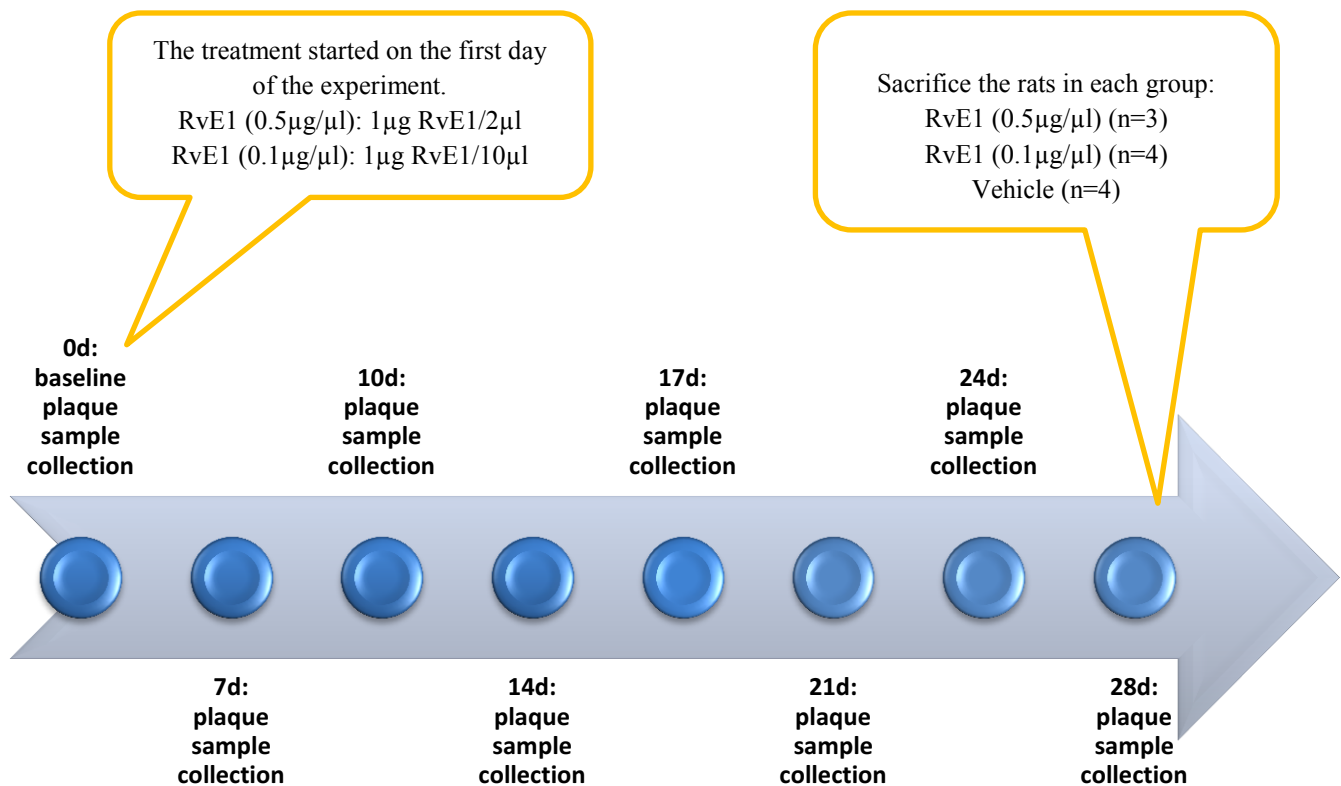


Figure 3.1: Experimental Timeline. Sixteen rats were assigned to four groups, but one rat in the RvE1 (0.5µg/µl) group was excluded from the study because of accidental anesthesia death (no ligature: n=4, ligature + Vehicle: n=4, ligature + RvE1 (0.1µg/µl): n=4, ligature + RvE1 (0.5µg/µl): n=3). The period of this experiment was four weeks. All rats had bilateral maxillary second molars ligated except the rats in the no ligature group. Rats in the no ligature group were sacrificed at baseline, and other rats were sacrificed at the end of the experiment. RvE1 (0.1µg/µl), RvE1 (0.5µg/µl), or vehicle (10% ethanol) was applied on the ligated teeth of rats in the different groups three times a week (M, W, F). Subgingival plaque samples were collected twice a week (M, F).

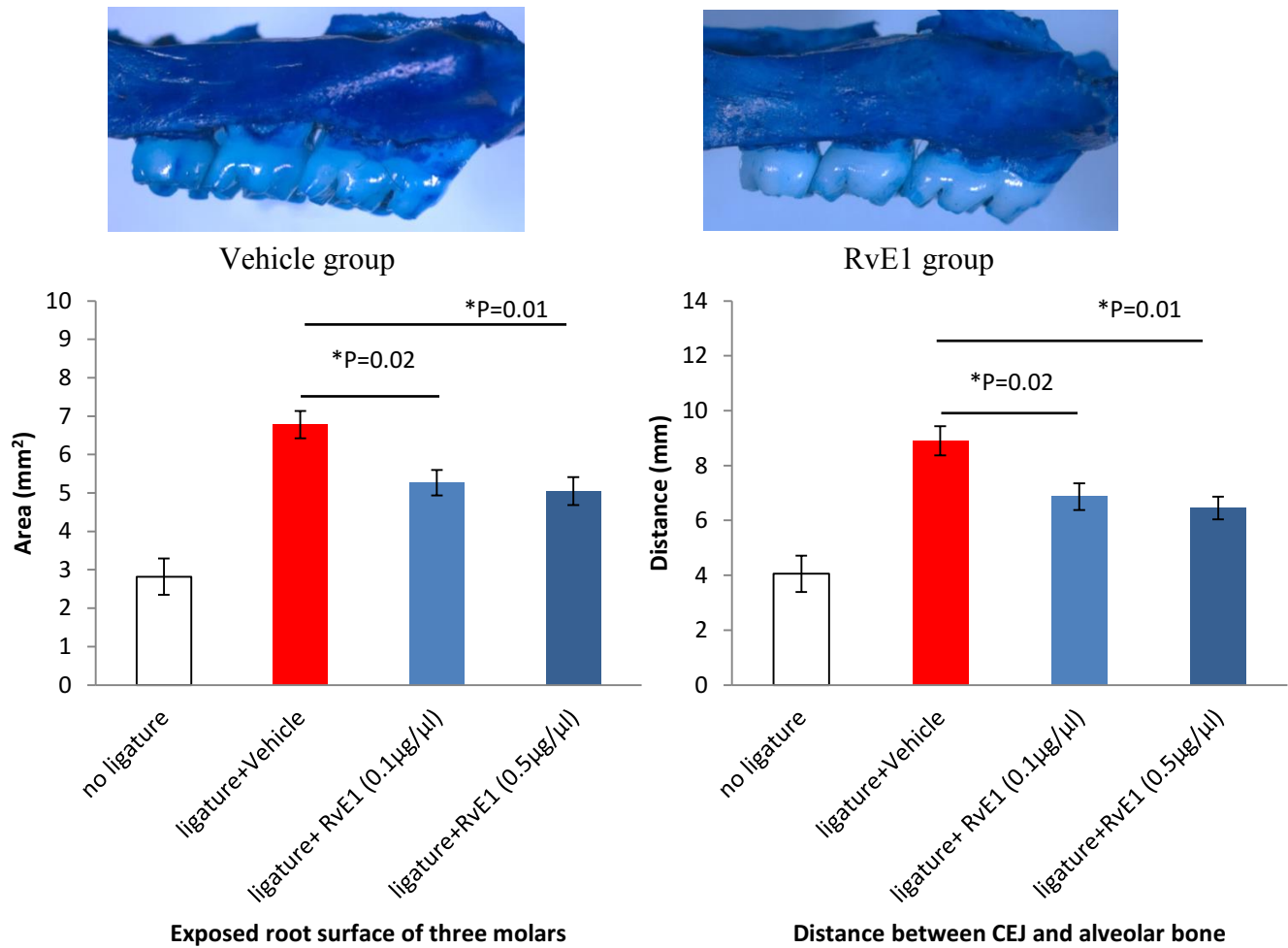


Figure 3.2: Bone morphometric analysis. The exposed root surface of three molars in the RvE1 groups (RvE1(0.1µg/µl) or RvE1 (0.5µg/µl)) was significantly smaller than that in the Vehicle group. The distance between CEJ and alveolar bone in the RvE1 groups was also significantly shorter than that in the Vehicle group. RvE1 application significantly prevented alveolar bone loss. The p-value was calculated by pair-wise t-test following one-way analysis of variance (ANOVA).

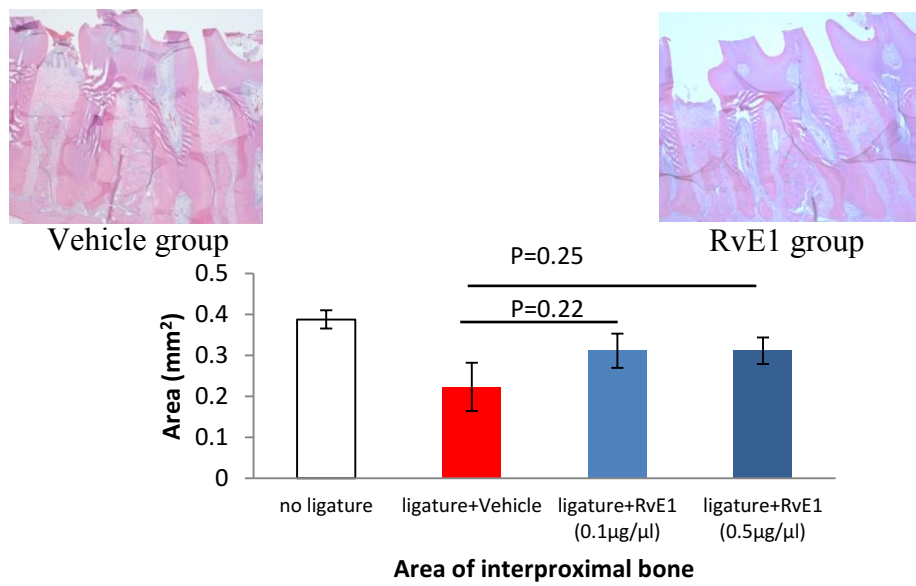


Figure 3.3: Histological area of interproximal bone. Area of interproximal bone in the RvE1 groups was greater than that in the Vehicle group ($p = 0.2$). The p -value was calculated by pair-wise t -test following one-way analysis of variance (ANOVA).

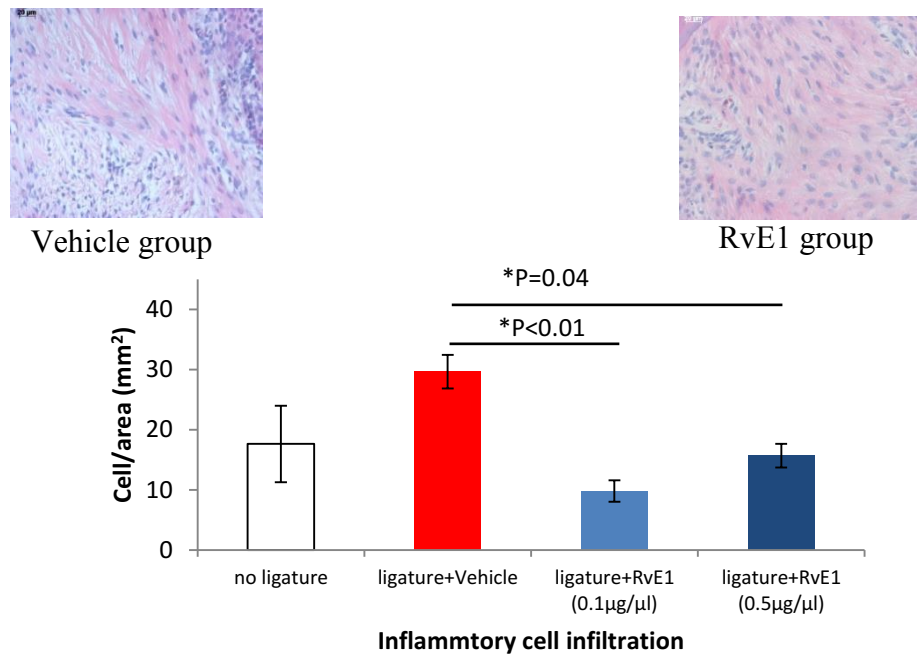


Figure 3.4: Inflammatory cell infiltrate. Inflammatory cell count in the RvE1 groups was significantly lower than that in the Vehicle group. There was no significant difference of inflammatory cell count between the RvE1 (0.1µg/µl) group and the RvE1 (0.5µg/µl) group. The p-value was calculated by pair-wise t-test following one-way analysis of variance (ANOVA).

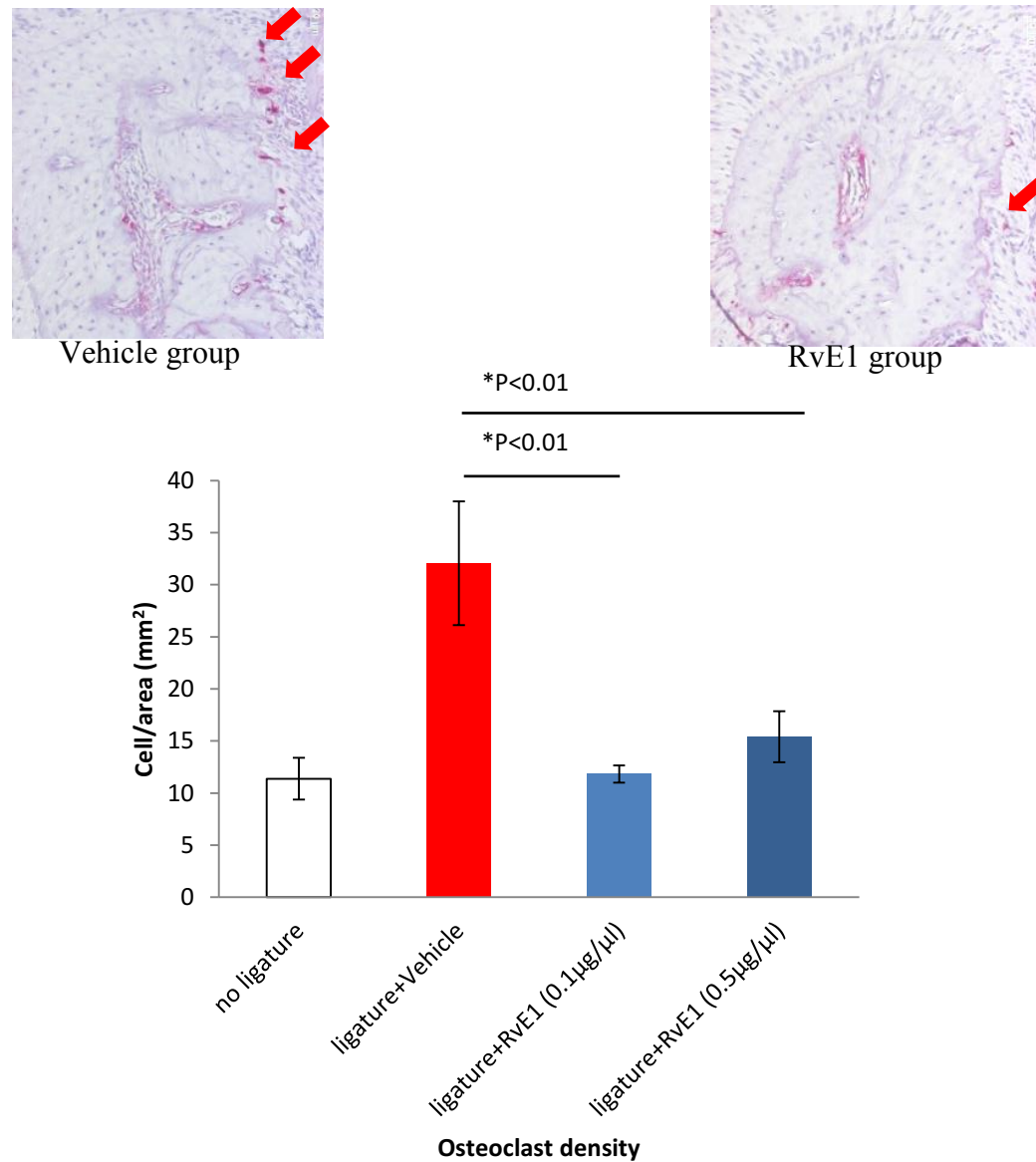


Figure 3.5: Osteoclast density. Osteoclast density was defined by dividing active osteoclast count (TRAP positive multinucleated osteoclasts) around the interproximal bone by the total area of the interproximal bone. The osteoclast density in the RvE1 groups (RvE1 (0.1µg/µl) or RvE1 (0.5µg/µl)) was significantly lower than that in the Vehicle group. Osteoclast density of the RvE1 (0.1µg/µl) group was not significantly different from that of the RvE1 (0.5µg/µl) group. The p-value was calculated by pair-wise t-test following one-way analysis of variance (ANOVA).

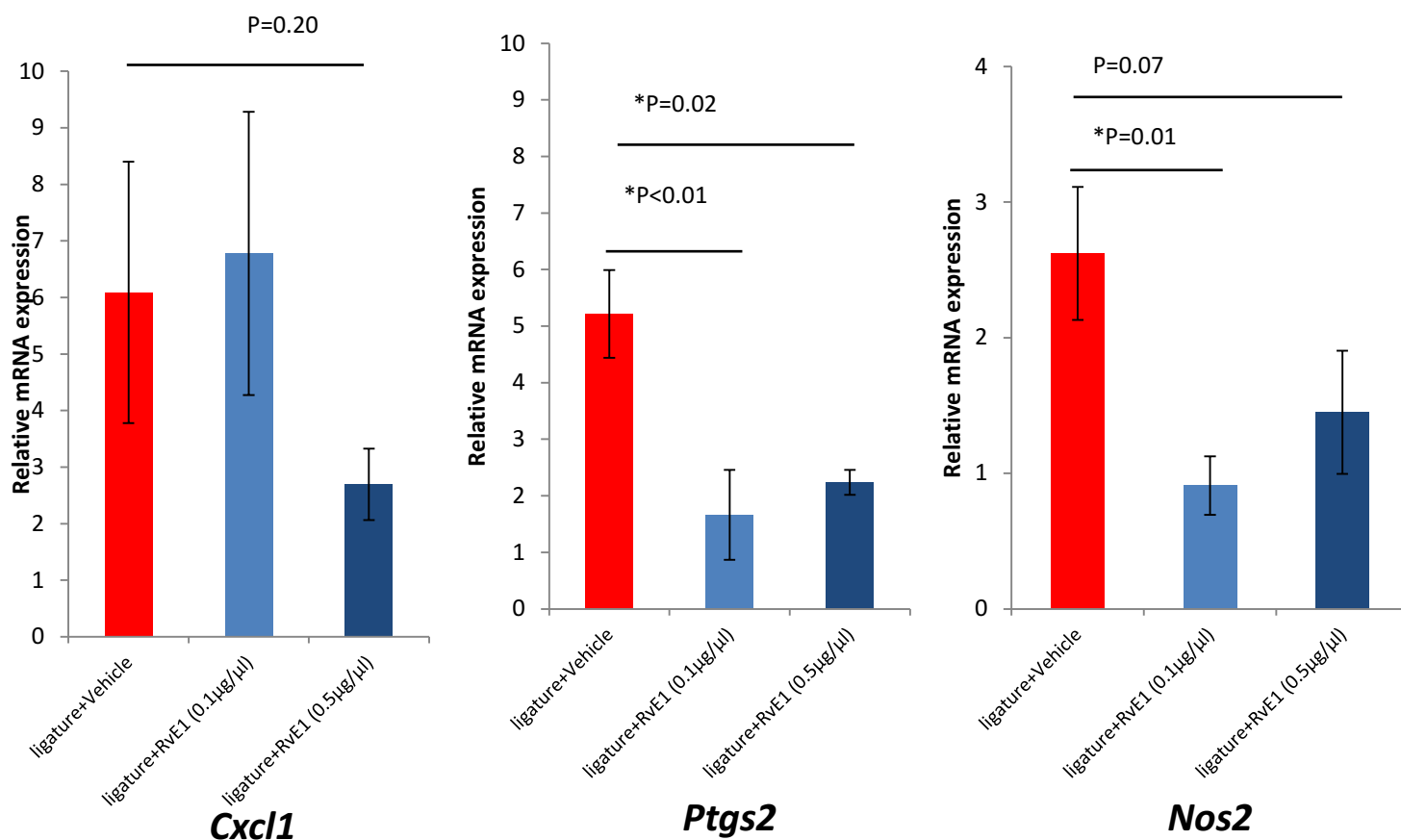


Figure 3.6: Relative quantity of inflammation-related gene expression ($2^{-\Delta\Delta CT}$). Relative mRNA expression ($2^{-\Delta\Delta CT}$) was used to analyze the gene expression levels of inflammation related genes in different groups. There was no significant difference in *Cxcl1* gene expression between the three groups. Gene expression of *Ptgs2* in the RvE1 groups (RvE1 (0.1 µg/µl) or RvE1 (0.5 µg/µl)) was significantly lower than that in the Vehicle group. Gene expression of *Nos2* in the RvE1 (0.1 µg/µl) group was significantly lowered than that in the Vehicle group. The p-value was calculated by pair-wise t-test following one-way analysis of variance (ANOVA).

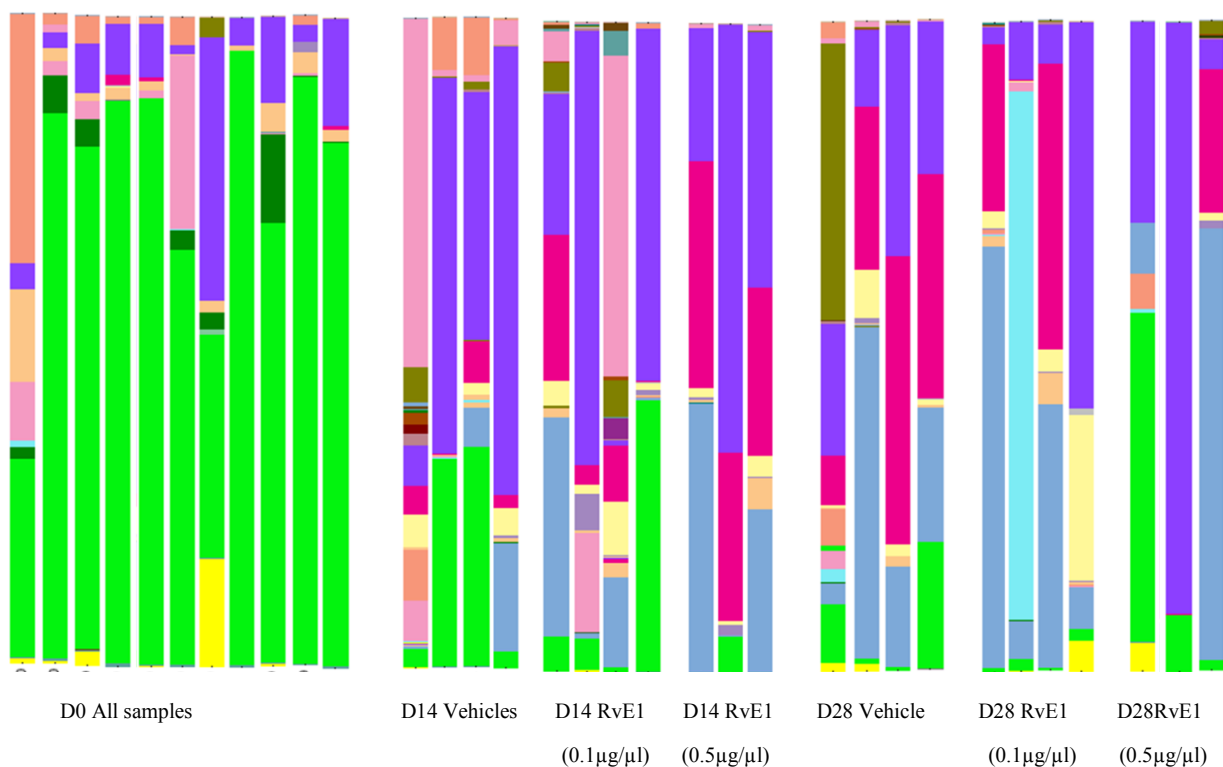
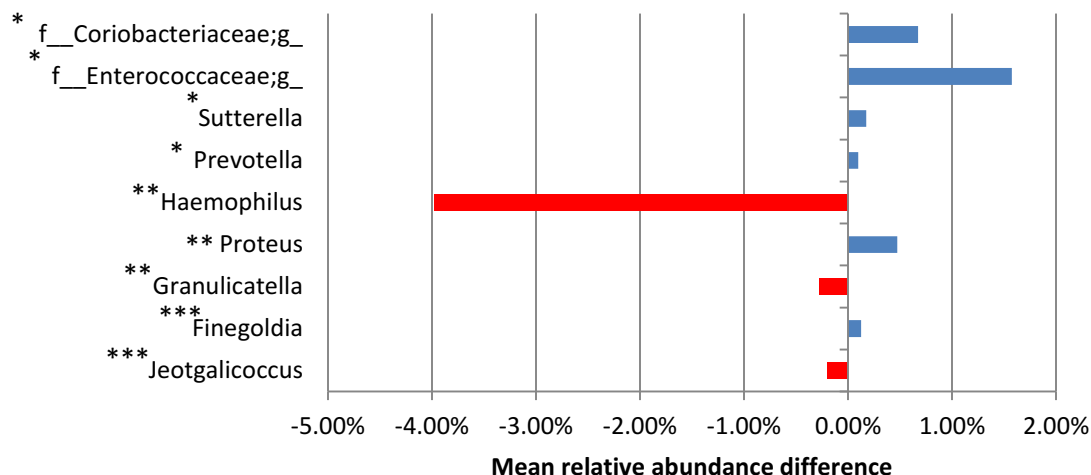


Figure 3.7: Taxonomic composition of the subgingival microbiota (genus level).

Taxonomic compositions of eleven samples in three groups are presented (Vehicle: n=4, RvE1 (0.1 µg/µl): n=4, RvE1 (0.5 µg/µl): n=3). The dynamics of the subgingival microbiota composition at different time points (day 0, 14, 28) was recorded. *Rothia* species was dominant at baseline. *Streptococcus* and *Lactobacillus* species had larger relative abundance than did other species after disease induction. Complete information of color coding genera can be found in Appendix 1.

A

D14 RvE1(0.1µg/µl) vs. Vehicle



B

D14 RvE1(0.5µg/µl) vs. Vehicle

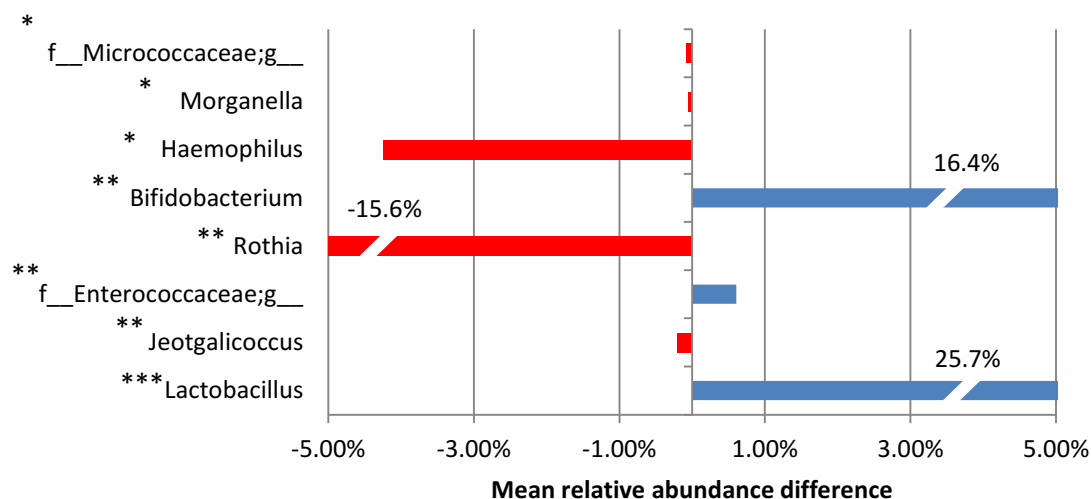


Figure 3.8: Mean difference of taxonomic relative abundance between RvE1 and Vehicle on day 14. Mean relative abundance difference between the RvE1 group (0.1µg/µl- Panel A, or 0.5µg/µl- Panel B) and the Vehicle group was calculated by subtracting mean relative abundance of the Vehicle group from mean relative abundance of the RvE1 group. Positive values indicate the mean relative abundance of the RvE1 group was larger than the relative abundance of the Vehicle group. The family name (f__) was listed for the OTUs with unknown genus. The p-values were calculated by Student's t test (***P<0.1; **0.1≤P<0.2; *0.2≤P<0.3).

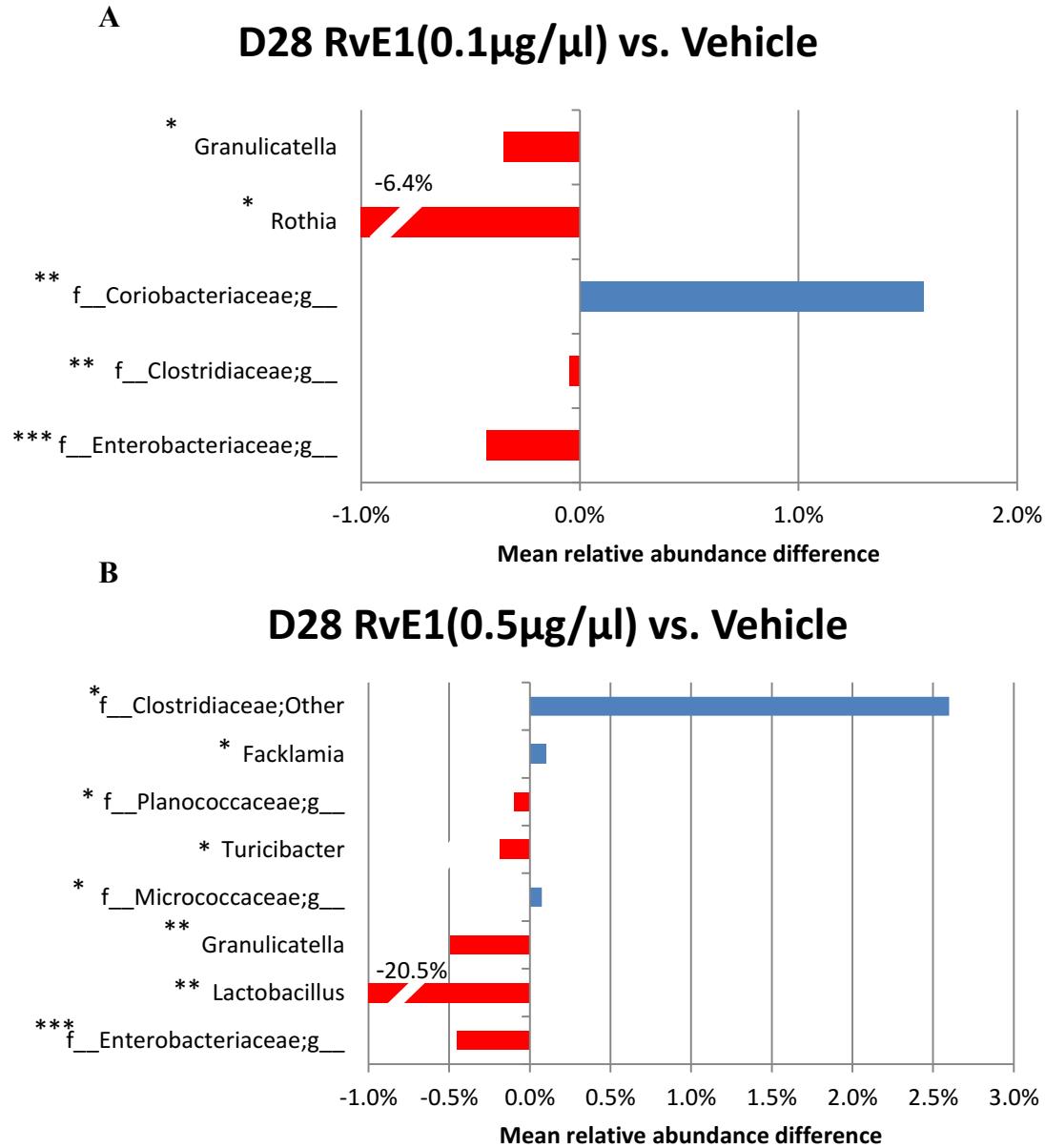


Figure 3.9: Mean difference of taxonomic relative abundance between RvE1 and the Vehicle on day 28. Mean relative abundance difference between the RvE1 group (0.1 μ g/ μ l- Panel A, or 0.5 μ g/ μ l- Panel B) and the Vehicle group was calculated by subtracting mean relative abundance of the Vehicle group from mean relative abundance of the RvE1 group. Positive values indicate the mean relative abundance of the RvE1 group was larger than that of the Vehicle group. Most of the genera had smaller relative abundance in the RvE1 group. The family name (f__) was listed for the OTUs with unknown genus. The p-values were calculated by Student's t test (*** $P < 0.1$; ** $0.1 \leq P < 0.2$; * $0.2 \leq P < 0.3$).

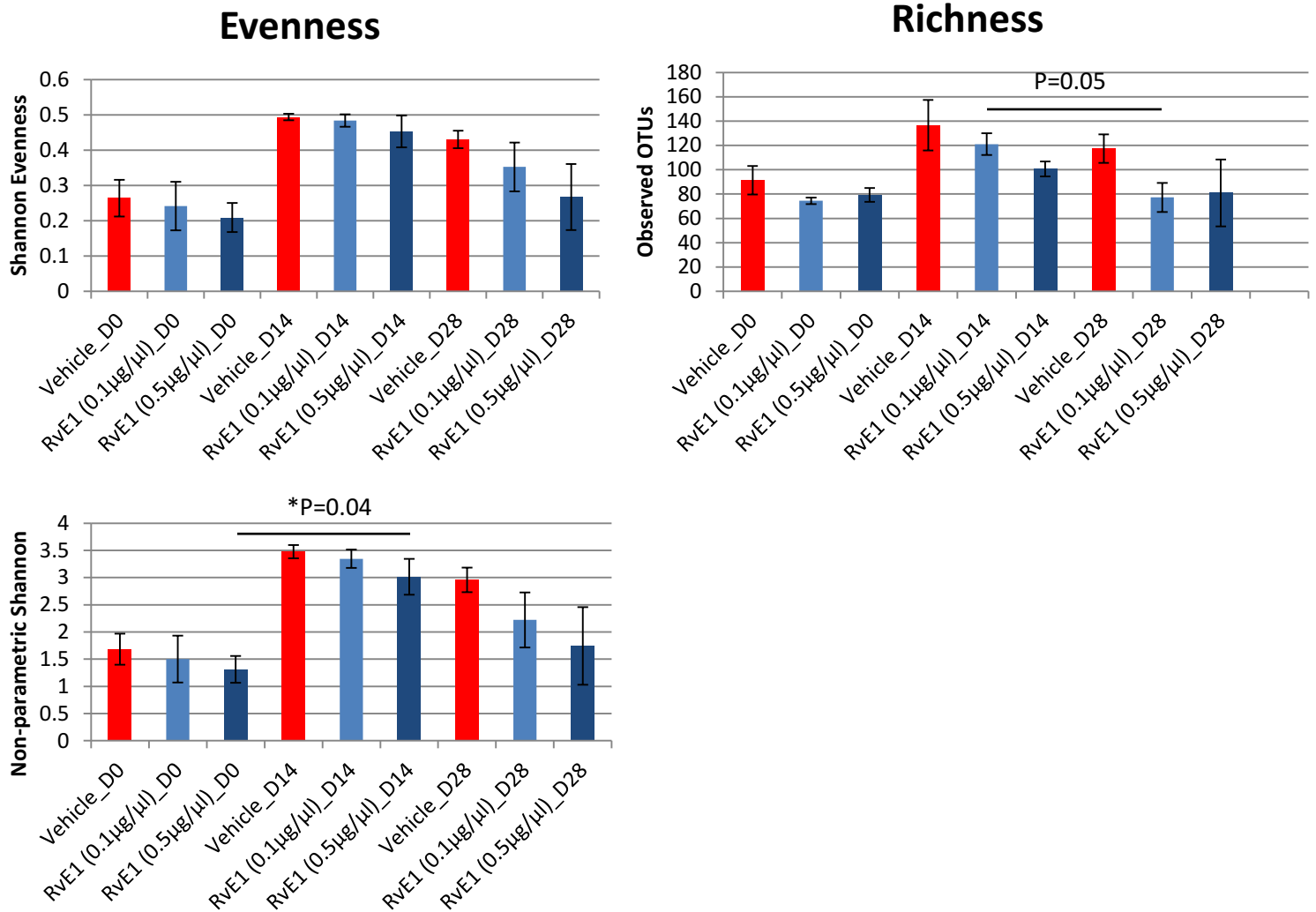


Figure 3.10: Alpha diversity of subgingival microbial community. The differences in Shannon evenness, non-parametric Shannon index or number of observed OTUs in the same group at different time points (d0 vs. d14, d14 vs. d28) were calculated (Student's t-test). Generally, the richness and evenness of microbial community were the highest on day 14. The evenness and richness of the Vehicle group were higher than the RvE1 groups (RvE1 (0.1µg/µl) or RvE1 (0.5µg/µl)) at all three time points. The results indicate that inflammation in the local environment in the Vehicle group might be more severe than that in the RvE1 groups as more species are expected in an inflammatory environment compared to an uninfamed environment.

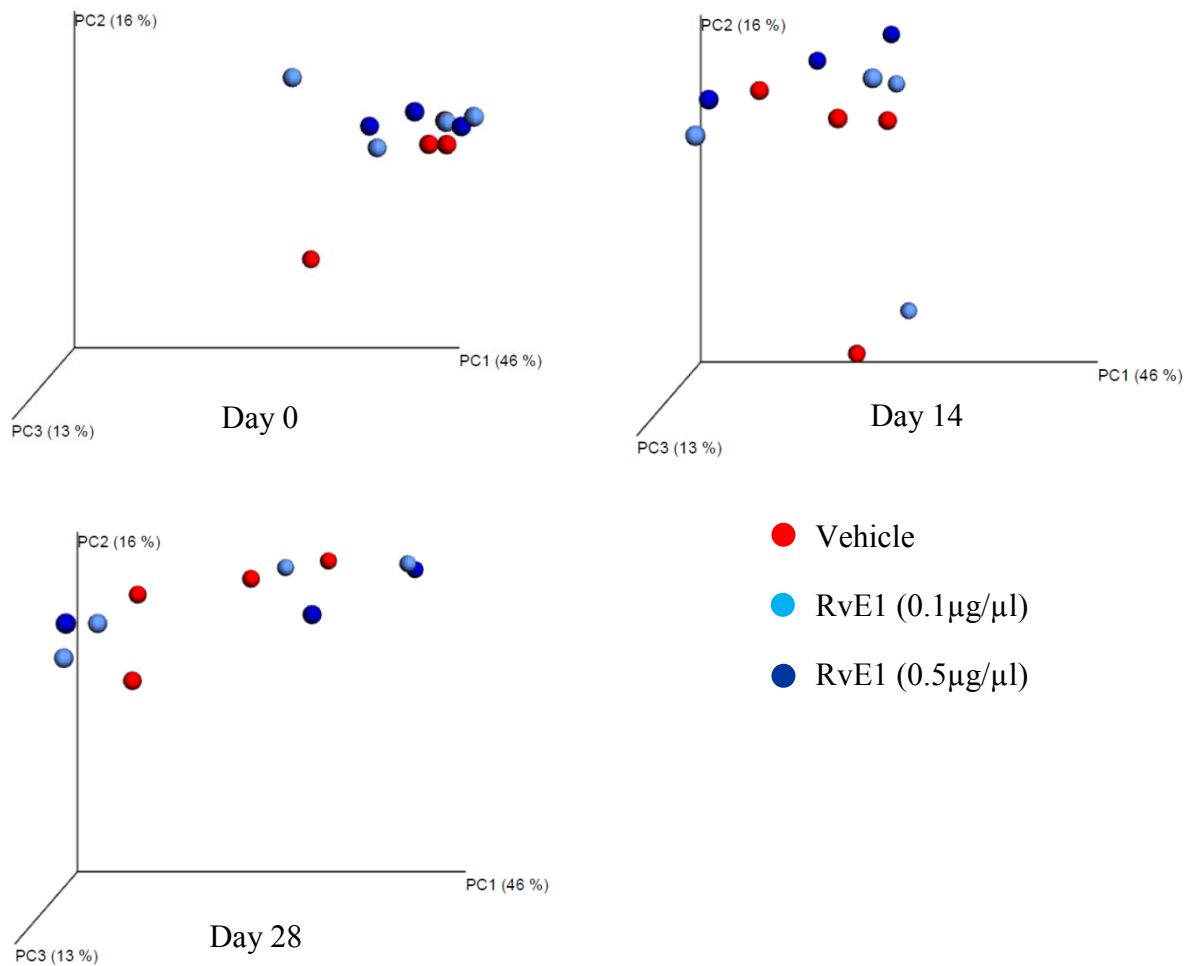


Figure 3.11: Principal coordinate analysis (PCoA). Principal coordinate analysis plots depicting distances among microbial communities based on qualitative community metrics reveal that there were significant shifts of microbial communities during disease progression. At the end of the experiment, microbial communities in three groups were not separated clearly. However, the communities within the Vehicle group appeared to be closer to each other than did communities of other two groups. The profile indicated the Vehicle group had smaller beta diversity (diversity between communities) than did other two groups. Reduced beta diversity is one of the characteristics of microbial community in periodontitis. Larger values of each component (PC) explain more of the variance in the data.

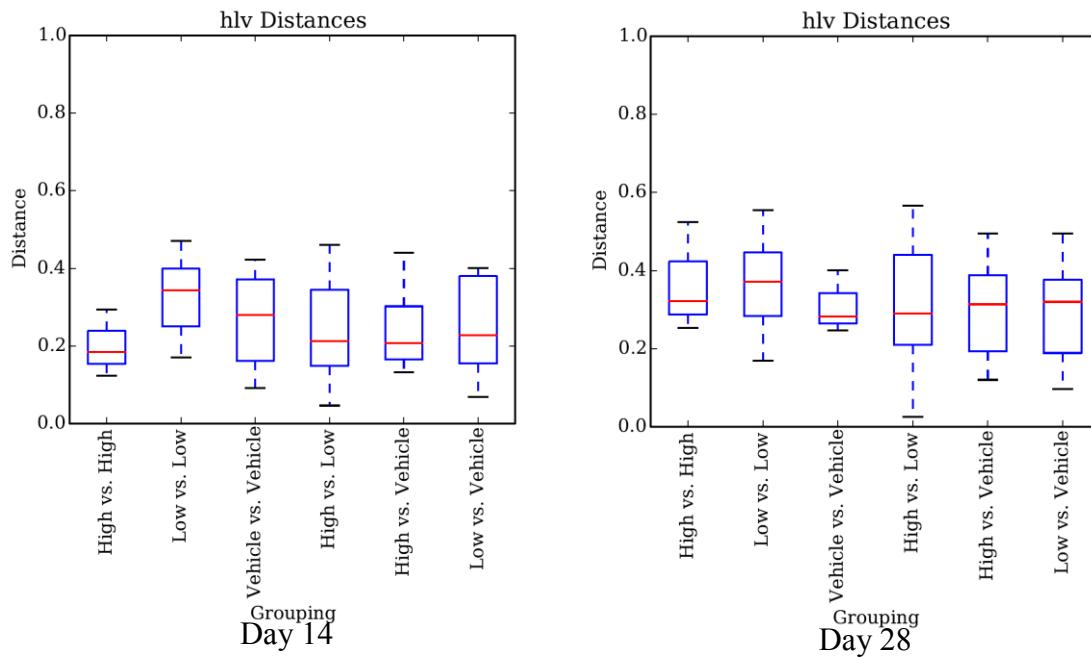


Figure 3.12: Beta diversity of subgingival microbial community at different time points. The mean distance between communities of the Vehicle group became shorter than that of other two groups at the end of the experiment (day 28). This result is consistent with principal coordinate analysis plot. The distances were calculated by weighted UniFrac (High: RvE1 (0.5 μ g/ μ l) group, Low: RvE1 (0.1 μ g/ μ l) group, Vehicle: Vehicle group).

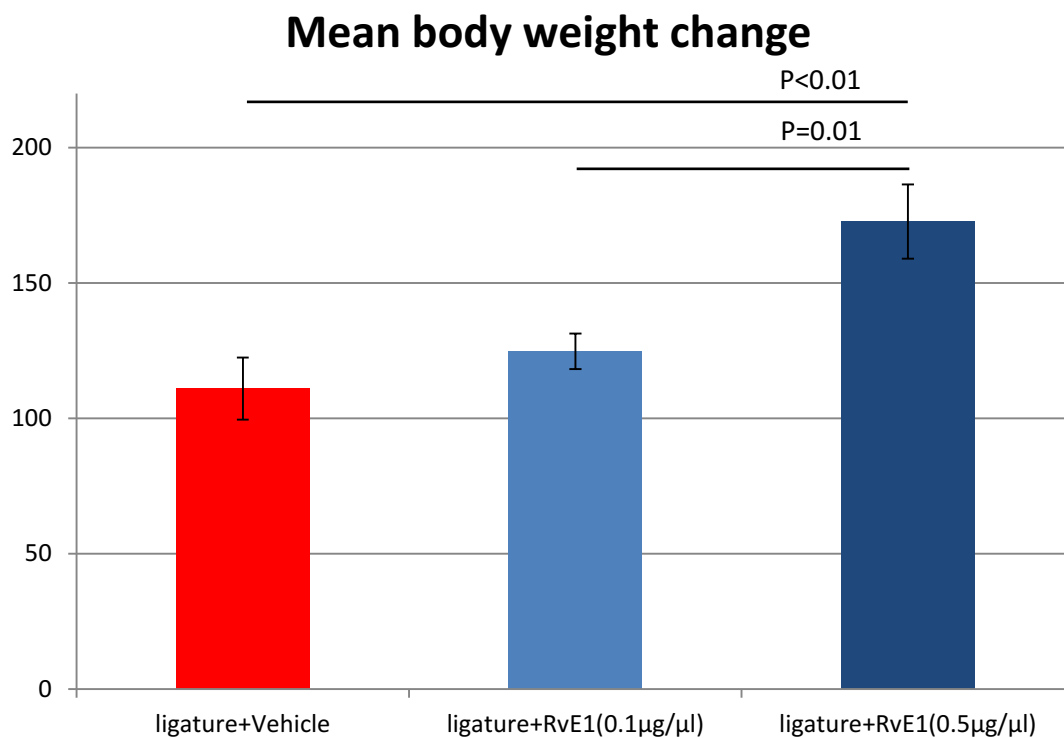


Figure 3.13: Mean body weight change during the experiment. Body weight change was defined as the body weight of the animal at baseline subtracted from the body weight of the animal at the end of the experiment (the 4th week). The mean body weight change of rats in the RvE1 (0.5µg/µl) group was significantly larger than the mean body weight change of rats in the RvE1 (0.1µg/µl) group and the Vehicle group.

Discussion

Prophylactic RvE1 treatment did not completely stop alveolar bone loss induced by ligature placement, but prophylactic RvE1 treatment significantly inhibited alveolar bone loss compared to vehicle application (Fig. 3.2). The prevention of alveolar bone loss was associated with the inhibition of inflammatory cell infiltration and the reduction of osteoclast activity following RvE1 application (Fig. 3.4, 3.5). The results confirmed the impact of RvE1 on the inhibition of neutrophil infiltration in the inflammatory environment (Arita et al., 2005b, Hasturk et al., 2007) and the inhibition of osteoclast differentiation (Gao et al., 2013). The gene expression of *Ptgs2* and *Nos2* in the gingival tissue was significantly inhibited by RvE1 application (Fig. 3.6). However, RvE1 application did not inhibit the expression of *Cxcl1* gene significantly. The result of limited change of *Cxcl1* gene expression following RvE1 application might be related to the acute nature of the insult as opposed to the chronic lesion in Chapter 2. Resolvins do not inhibit inflammation. Rather, they provide a feed forward, receptor agonist driven enhancement of resolution. Since *Cxcl1* expression is critical in the acute phase and the other two genes are expressed later, the lack of impact of RvE1 on *Cxcl1* in the acute phase should not be unexpected. Different concentrations of RvE1 (0.1 or 0.5 $\mu\text{g}/\mu\text{l}$) did not have significantly different impact on prevention of alveolar bone loss, inhibition of inflammatory cell infiltration, inhibition of osteoclastogenesis, and reduction of expression of inflammation related genes in gingival tissue.

The taxonomic composition of the subgingival microbiota at baseline was significantly different from that following ligature placement (Fig. 3.7). Generally,

Rothia was the dominant genus with the highest relative abundance (72.4 ± 6.6 %) and *Streptococcus* species had the second largest relative abundance (9.8 ± 3.4 %) at baseline. Similar results were reported earlier in the taxonomic composition of supragingival biofilm in rats (Manrique et al., 2013). *Bifidobacterium*, *Lactobacillus*, or *Streptococcus*, were generally the dominant genera during disease progression. Considering the variation of relative abundance and limited sample size, a less conservative significant level ($p < 0.3$) was chosen to find the potential genera representing subgingival microbiota in different groups. While comparing the mean relative abundance of different genera between the RvE1 groups (0.1 or 0.5 $\mu\text{g/ml}$) and the Vehicle group, several genera had a relatively more significant difference ($p < 0.3$) than other genera (Fig. 3.8, 3.9). Most of the genera on day 14 were different from the genera on day 28. The results indicate the subgingival microbiota composition was dynamic during disease progression. For example, *Lactobacillus* genus had an inverse relationship in the three groups on day 14 and day 28 (Mean relative abundance \pm SEM: RvE1 (0.5 $\mu\text{g}/\mu\text{l}$) vs. RvE1 (0.1 $\mu\text{g}/\mu\text{l}$) vs. Vehicle- 28.9 ± 3 , 8.6 ± 4.9 , 3.3 ± 1.4 % on day 14; RvE1 (0.5 $\mu\text{g}/\mu\text{l}$) vs. RvE1 (0.1 $\mu\text{g}/\mu\text{l}$) vs. Vehicle- 7.4 ± 7.4 , 17.5 ± 10.7 , 27.9 ± 8.0 % on day 28). At the end of the experiment, these genera, which had more significant difference of relative abundance between groups than did other genera, generally had small relative abundance ($< 1\%$) and had reduction of relative abundance in the RvE1 groups (Fig. 3.9.a&b). Two of these genera were found in both RvE1 groups. One genus, which belongs to *Enterobacteriaceae* family, could not be assigned to any known genus in the reference genome. Another genus, *Granulicatella*, has species in the commensal oral bacteria found in dental plaque (Mikkelsen et al.,

2000). The *Granulicatella* species are also related to refractory periodontitis (*Granulicatella elegans*) (Colombo et al., 2012), acute dental abscess (*Granulicatella adiacens*) (Robertson and Smith, 2009), endodontic infection (*Granulicatella adiacens*) (Siqueira and Rocas, 2006) and endocarditis (*Granulicatella adiacens*, *Granulicatella elegans*) (Cargill et al., 2010) in humans.

The richness and evenness of the microbial community in the Vehicle group were higher than in the RvE1 groups following disease induction. Although the richness and evenness of the microbial community in the Vehicle group were also higher than in the RvE1 groups at baseline, the difference between the groups became more significant following disease induction (Fig. 3.10). The dose effect of RvE1 appeared to play a role in affecting community diversity given the richness and evenness of microbial community in the RvE1 group (0.5µg/µl) was lower than these in the RvE1 group (0.1µg/µl). While considering the dynamics of community diversity at different time points, the evenness and richness were highest on day 14 and lowest on day 0. Given the evenness and richness of microbial community were positively related to diseased state of periodontitis (Abusleme et al., 2013, Shi et al., 2015), the dynamics of community diversity might represent the change of inflammation process in experimental periodontitis. The most severe inflammation happened within the first two weeks, but then the inflammation decreased gradually. The inhibition of inflammation by RvE1 appeared to repress the growth of multiple species resulting in the decrease of richness and evenness of microbial community.

The subgingival microbiota of all groups shifted following ligature placement (Fig. 3.11). At the end of the treatment, the mean distance representing the diversity between samples was the lowest in the Vehicle group (Fig. 3.13). The reduced beta diversity in the Vehicle group could be linked to the unresolved inflammation. Taken together, the change of subgingival microbiota was different between the RvE1 groups and the Vehicle group. The results indicate that the prevention of local environment change induced by experimental periodontitis might be associated with the changes of diversity and taxonomic composition in subgingival microbiota.

RvE1 and other SPMs have shown the analgesic properties by regulating specific receptors, such as transient receptor potential cation channel subfamily V member (TRPV), dependent nociception (Xu et al., 2010, Bang et al., 2010). RvE1 treatment also prevented body weight loss in the colitis mice model by controlling acute inflammation (Arita et al., 2005b). It is interesting to observe the correlation between RvE1 application and body weight change of the animals (Fig. 3.13). RvE1 treatment might affect the gain of weight in animals by controlling pain and inflammation induced by ligature placement. It has been demonstrated that body weight change was affected by post-operative pain in animal model (Jablonski et al., 2001, Brennan et al., 2009). However, more studies have to be conducted to investigate the correlation between body weight change and trauma induced by the ligature placement in experimental periodontitis model.

CONCLUSIONS

Resolvin E1 treatment of existing ligature induced periodontitis significantly regenerates lost alveolar bone. Prophylactic resolvin E1 treatment significantly prevents alveolar bone loss in ligature induced periodontitis rat model. The limited shift of subgingival microbiota in experimental periodontitis following resolvin E1 application was associated with reduced inflammation and repressed osteoclast activity in the local environment. The results of this experimental periodontitis model suggest that changes in the composition of the subgingival microbiota emerge as a result of inflammation.

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Appendix 1. Color coding taxa

Color	Taxonomy
	Unassigned;Other;Other;Other;Other; Other
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;Other; Other
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae; g__
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae; g__Actinomyces
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Brevibacteriaceae; g__Brevibacterium
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae; g__Corynebacterium
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dermabacteraceae; g__Brachybacterium
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae; Other
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae; g__
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae; g__Arthrobacter
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae; g__Kocuria
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae; g__Rothia
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Yaniellaceae; g__Yaniella
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae; g__Bifidobacterium
	k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae; g__
	k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae; g__Adlercreutzia
	k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae; g__Atopobium
	k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae; g__Collinsella
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__; g__
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae; g__Bacteroides
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae; g__Paludibacter
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae; g__Porphyromonas
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae; g__Prevotella
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7; g__
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]; g__[Prevotella]
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae; g__Capnocytophaga
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae]; g__
	k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other; Other
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;Other; Other
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__; g__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae; Other
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae; g__Bacillus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae; g__

	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__ Sporosarcina
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;Other
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__ Jeotgalicoccus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__ Staphylococcus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae;g__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;Other;Other
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__g__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__ Aerococcus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__ Facklamia
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__ Granulicatella
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;Other
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__ Enterococcus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__ Vagococcus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__ Lactobacillus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__ Pediococcus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__ Lactococcus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__ Streptococcus
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__ Turicibacter
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__g__
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;Other
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__ Clostridium
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__ SMB53
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__ Blautia
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__ Catonella
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__ Coprococcus
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__ Dorea
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__ [Ruminococcus]

	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__ Peptostreptococcus
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__ Oscillospira
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__ Ruminococcus
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__ Dialister
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__ Veillonella
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__ Finegoldia
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__ Parvimonas
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__ Peptoniphilus
	k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__
	k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__ Allobaculum
	k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__ Bulleidia
	k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__ [Eubacterium]
	k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__ Fusobacterium
	k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__
	k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__ Leptotrichia
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__ Sutterella
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__ Lautropia
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__ Neisseria
	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacteriales;f__Campylobacteraceae;g__ Campylobacter
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Cardiobacteriales;f__Cardiobacteriaceae;g__ Cardiobacterium
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__ Other
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__ Morganella
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__ Proteus
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__ Actinobacillus
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__ Aggregatibacter
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__ Haemophilus
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__ Pseudomonas

	k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__ Treponema
	k__Bacteria;p__TM7;c__TM7-3;o__I025;f__Rs-045;g__
	k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__ Akkermansia

